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PREPARATION AND PROPERTIES OF ENZYME POWDERS FOR ETHYLENE SYNTHESIS

by

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A THESIS
SUBMITTED TO THE FACULTY OF GRADUATE STUDIES
IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE
DEGREE OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF PLANT SCIENCE

EDMONTON, ALBERTA

AUGUST 1966

UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled "Preparation and Properties of Enzyme Powders for Ethylene Synthesis" submitted by John Eveleigh Thompson in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

ABSTRACT

Elucidation of a biosynthetic pathway for the physiologically active gas, ethylene, has been the object of considerable experimentation. Many studies have been performed with intact organisms, tissue slices and more recently, subcellular particulate fractions. In an effort to obtain a simplified system for such pursuits, the present investigation was undertaken with the objective of preparing an enzyme powder active in ethylene production.

Ethylene biogenesis accompanies maturation and aging and hence a tissue in which the degree of senescence could be relatively easily assessed, was sought as a source for the enzyme system. Wax bean cotyledons, the short-lived first leaves of *Phaseolus vulgaris*, fulfilled this requirement. From these, and also from wax bean seedlings and oat seedlings, *Avena sativa*, enzyme powders capable of catalyzing ethylene production in the presence of appropriate substrates and cofactors were obtained.

For preparation of the powders, a tissue homogenate was subjected to differential centrifugation, and the fraction isolated at 35,000 g was suspended in deionized water and frozen and thawed for disruption of particles. Subsequent ammonium sulphate precipitation and lyophilization yielded a crude enzyme system. Removal of lipids and subjection of the remaining residue to ammonium sulphate fractionation resulted in the preparation of a partially purified enzyme powder that was able to catalyze ethylene formation.

β-alanine, together with pyridoxal phosphate and ferric ion for transamination and thiamine pyrophosphate and magnesium ion for decarboxylation, when added to the enzyme powder with ATP, yielded ethylene. When

β-alanine-2-^{14}C was added to a complete reaction mixture, radioactive ethylene was obtained. Intermediates arising from this conversion were isolated and identified by paper and thin layer chromatography.

Non-enzymatic production of the volatile by a heat-catalyzed Snell reaction among β-alanine, pyridoxal hydrochloride and cupric ion provided further evidence for β-alanine as a precursor of ethylene.

Substrate, cofactor and radioactive tracer studies were all consistent with a pathway for ethylene synthesis that involves conversion by a transaminase of β-alanine to malonic semialdehyde, and thence transformation through β-hydroxypropionic acid and acrylic acid to ethylene.

ACKNOWLEDGMENTS

I wish to express my deep and sincere gratitude to Dr. Mary Spencer for her valuable guidance throughout this investigation. Her helpful suggestions, constructive criticisms, encouragement and sincere interest in the research problem were a constant source of inspiration.

Thanks are extended to Dr. E. Cossins, Dr. S. Zalik, Dr. H.B. Collier and Dr. J. Tuba for their interest and encouragement. I am also grateful to Messrs. A. Olson, R. Stinson, J. Vose and H. White for their helpful suggestions, criticisms and support.

I am indebted to Mrs. Nora Everaars and Mr. Thomas Tribe for excellent technical assistance. A special note of thanks for typing the thesis is extended to Miss Phoebe Hines. In addition, I wish to express my deep appreciation to my wife, Janet, for her wonderful understanding and encouragement.

I thank the Ogilvie Flour Mills Co. Ltd. for a fellowship of two years duration.

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LIST OF ABBREVIATIONS

1. ATP: Adenosine triphosphate
2. CoA: Coenzyme A
3. CPM: Counts per minute
4. DPM: Disintegrations per minute
5. NAD: Nicotine adenine dinucleotide
6. NADP: Nicotine adenine dinucleotide phosphate
7. TPP: Thiamine pyrophosphate
8. 2,4-D: 2,4 dichlorophenoxyacetic acid

Chapter I

INTRODUCTION

The diversity of organisms producing ethylene is striking. Ripening fruits were among the first tissues found to evolve the gas; in 1934 ethylene was chemically identified as an emanation from ripening apples (1). Now it is known that a multitude of fruits produce the volatile. For example, some recent studies on ethylene include work done with avocado (2),(3), cantaloupe (4),(5), pineapple (6), mangoes (7), bananas (8), tomatoes (9), honeydew melons (10), and citrus fruit (11). Quantities of ethylene evolved by fruits range from 0.05 μl per Kgm. per hr. for valencia oranges to 350 for passion fruit (12),(13).

Penicillium digitatum, a fungus that is the common green mold of citrus fruit, is also a high producer of ethylene (14),(15),(16). Other fungi known to give off the gas are Alternaria citri (17), Blastomyces dermatididis (18), and Fusarium oxysporum (19). In addition the bacterium Pseudomonas solancearum has been found to yield ethylene in substantial quantities (20).

That ethylene is evolved from intact plants is substantiated by its presence in the volatile emanations from cotton plants (21), castor bean seedlings (22), oat seedlings (23), and pea seedlings (24). The gas has been obtained from flowers (13), leaves (25), and pea epicotyls (26), indicating that some parts of plants are producers of the volatile as well. It has been suggested that all parts of plants may form ethylene.

Recent investigation has shown that ethylene is produced by animal tissue. Chandra and Spencer (27) observed the olefin to be an emanation from subcellular particles of rat liver and rat intestinal

mucosa. Later, subcellular particles from beef heart were also found to be producers of the gas (28). Moreover, ethylene was detected as a component of the exhaled respiratory gases of the human species at up to 10 parts per 10^9 (29). Collections of the exhaled respiratory volatiles of rats also revealed the presence of ethylene (30). Evidence does indeed indicate that ethylene genesis is a general phenomenon of life.

It is apparent that ethylene evolution accompanies maturation and aging of tissues. Studies with ripening tomatoes indicated increased ethylene evolution (31) accompanying a peak in carbon dioxide production; the latter constituted the climacteric respiratory rise. A peak in ethylene production and the climacteric rise are characteristic of many fruits during ripening. It is interesting that with the onset of climacteric behaviour, fruit tissues showed marked signs of deterioration. For example, a substantial increase in the rate of endogenous solute leakage was observed at the onset of climacteric respiration in ripening banana and avocado tissue (32,(33). Further, the rate of leakage increased progressively in accordance with the respiratory rise. Such losses of permeability barriers could reflect alterations in compartmentalization that affect relationships between enzymes and substrates responsible for ethylene production. Inasmuch as increased permeability accompanies senescence, the former may account for the association of ethylene evolution with aging of tissue. The connection between senescence and ethylene production is prevalent in tissue other than ripening fruit, for Akamine (34) obtained a peak in ethylene production (3400 μl per Kgm of tissue per hour) from Vanda Orchid blossoms when 97 percent of the blossoms had faded.

Tissues in a diseased condition, which simulates senescence of

tissue to the extent that there is a structural breakdown, produce substantial quantities of ethylene. Introduction of M-1 Sarcoma under the skin of rats caused increased ethylene production during the initial period of tumour development (30). Smith, Meigh and Parker (35) observed increased ethylene evolution by carnations infected with spores of Botrytis. Damage to plant tissues appears to be instrumental in raising the rate of production of ethylene, and in this instance increased production probably reflected a breakdown by the invading organism of host tissues. Dissection of cantaloupe caused increased respiration and ethylene emanation (36); accelerated evolution of the volatile may have been a result of wounding incurred by the tissue.

Application of phenoxyacetic compounds to bean petiole explants promoted leaf abscission and also augmented ethylene production (37). It has been suggested (37) that endogenous ethylene may play a role in abscission processes. Sub-lethal concentrations of both 2,4-D and indole acetic acid accelerated evolution of ethylene by the cotton plant (38).

Some interesting studies have been carried out to assess the effects of gamma irradiation of tissue on ethylene production. Young (3) detected increased respiration and ethylene yield when avocado tissue was exposed to cobalt-60 gamma rays for 100 minutes. Only preclimacteric fruit responded. It was suggested that climacteric tissue had possibly attained by natural aging the condition induced in preclimacteric fruit by irradiation. Similar increases in ethylene yields were obtained by gamma irradiation of lemons (11) and pea epicotyl tissue (26). The boost in ethylene production on irradiation of tissue could reflect some changes in enzyme-substrate relationships normally caused by aging, or the

ethylene may be an artifact caused by the direct action of ionizing radiation on components of the tissue. Gamma irradiation of pea epicotyl tissue in a nitrogen atmosphere failed to stimulate ethylene evolution (26).

It is significant that the need for aging is extended to ethylene production on a subcellular level. Like intact fruit, isolated subcellular particles required partial disintegration before the ethylene-producing mechanism was stimulated, although the intact mitochondria did produce small amounts of the volatile. Simple aging of the subcellular fraction or sonication were effective as a means of disintegration for accelerated ethylene production (39).

Although ethylene may simply be a byproduct of aging, the fact that application of minute quantities of the volatile, of the order of 1 p.p.m., to fruits will stimulate climacteric (40) and nonclimacteric (17) respiration and induce ripening (41), suggests a more significant connection between the two phenomena. It is interesting to speculate that ethylene may in some way spark the aging process. Perhaps elucidation of a biosynthetic pathway for the volatile coupled with a knowledge of the mechanisms underlying its many effects, may give rise to a greater understanding of the aging process. As well, ethylene has economic importance for the fruit industry in view of its capacity to hasten ripening.

1. Biological Effects of Ethylene

Ethylene exhibits a wide variety of biological effects. Among the more interesting of these is its capacity to accelerate the onset of the climacteric respiratory rise (40) and induce fruit ripening (41). Ethylene has been denoted as a "ripening hormone" by some (42), (43).

It has also been found to hasten the latter stages of maturity of some dried fruits. For example, its application will promote loosening of hulls of pecans (44) and walnuts (45). Studies with honeydew melons (10) have indicated that ethylene may be part of a mechanism triggering the climacteric respiratory rise and possibly ripening. This theory is supported by other research (46) in which ethylene was applied at 1000 ppm to fruits harvested at a mature-green stage and stored at 20°C; those treated ripened earlier and more uniformly than those not treated.

It later became apparent (4) that fruit must acquire a certain minimum stage of development before responding characteristically to applied ethylene. In fruits that had completed one half of their growth prior to ethylene treatment, the induced climacteric closely resembled the natural climacteric. When fruits were treated at a younger stage however, climacteric behaviour was induced only after a time lag and ripening appeared late and was often ill defined. The magnitude of the induced climacteric in fruits having completed more than one half their growth, can be up to 50 percent greater than that occurring naturally, but the total respiratory increases in both instances are similar. If ethylene is the causative agent, it is reasonable to attribute this greater magnitude in the case of treated fruit to a simultaneous stimulation of all cells. A gradual stimulation of cells, effected by endogenous ethylene, would probably occur during a natural climacteric rise, since many fruits, for example cantaloupe, ripen from the inside out. Again, if ethylene is the causative agent, the required minimal stage of development for response to the inducing effects of the volatile may reflect a certain minimal level of endogenous ethylene and thereby indicate changes in metabolism that allow such an accumulation. It is significant in this

respect that some ethylene has been found present in immature cantaloupe fruits (5). In fact Burg (7) has reported the presence of ethylene in several fruits prior to the climacteric stage. Also pertinent is the observation that tissue slices from cantaloupe fruit harvested 30 days after anthesis produced ethylene much faster than did those from fruit harvested 15 to 20 days after anthesis (36). During 70 - 93 percent of total tomato fruit growth, ethylene production increased only 10 fold, but during ripening it increased 35 fold (47).

It has been calculated that the internal ethylene concentration in some fruits prior to commencement of climacteric respiration is well in excess of 0.1 ppm (41), which is the average minimum necessary to stimulate fruit ripening. Burg and Burg (7) found that application of ethylene to mangoes hastened ripening even after climacteric respiration had begun. These workers have in addition shown that the response of some fruits to ethylene is apparently related to the logarithm of the internal ethylene concentration (7).

In consideration of the association of ethylene evolution with climacteric respiration, it may be significant that application of solutions of the volatile to mitochondria isolated from rat liver and cauliflower buds increased the rate of swelling (48). Olson and Spencer (49) have observed an increased uptake of nucleotides by mitochondria from wax bean cotyledons in the presence of ethylene. Such evidence implicates that ethylene may achieve its effects through alteration of membrane permeability.

When freshly harvested high moisture wheat was treated with 100 ppm of ethylene, production of carbon dioxide increased (50). In addition the percentage germination of treated samples was superior to untreated.

Ethylene has also been effective in securing germination of seeds of buck thorn, high bush cranberry, honeysuckle and snow berry (51).

Similarities exist between the effects of ethylene and auxins on plants. There is evidence for a synergistic effect of auxin and ethylene causing increased shoot length (52), and moreover the fact that auxin stimulated ethylene production from roots, stems and leaves of different genera (53) suggests that the level of auxin regulates the production of ethylene. It has been proposed that since application of certain auxins will induce leaf abscission (37), consequent increased ethylene evolution constitutes a mechanism for abscission. Further, in view of the inhibition by actinomycin and puromycin of auxin-stimulated ethylene production, it has been postulated that the mechanism of action of growth substances constitutes activation of enzymes involved in ethylene biosynthesis (54).

Ethylene exhibits a host of additional effects; many of them are outlined by Jansen (12). Included is a triple response to the volatile by some plants such as etiolated pea seedlings; the response consists of leaf epinasty, swelling of the stem and inhibition of extensive growth. These effects of the volatile were used as bioassays before the advent of more sensitive techniques. That ethylene will serve as an anaesthetic (55), further demonstrates the diversity of its effects.

2. Biosynthesis of Ethylene

Considerable effort is being directed towards elucidation of a biosynthetic pathway for ethylene. Perhaps one of the more significant facts implicating production of the volatile to be enzymatic is the sensitivity of the ethylene-producing mechanism to temperature changes. Craft (56) found that ethylene production by cubes of tomato tissue was maximal at 33°C, retarded at 40°C and higher than at 40°C when the temper-

ature was lowered to 30°C. Boiling inactivated the ethylene-producing system in a subcellular fraction from tomatoes (57). As well, the subcellular fraction yielded less ethylene at 40°C and 50°C relative to controls at 25°C. Inactivation of the ethylene-producing system at 100°C and decreased activity at 40°C and 50°C are consistent with the usual behaviour of enzymes.

(a) Pathway Studies with Tissue Slices and Intact Organisms

Many of the studies on the pathway have been conducted with intact organisms or tissue slices. Such exploration has incorporated experiments with inhibitors and labeled and unlabeled substrates.

(i) Inhibitors

Inhibitor studies on ethylene production by apple tissue plugs were conducted by Burg and Thimann (58). Effective inhibitors included 2,4 dinitrophenol, fluoride, iodoacetamide, arsenite, fluoroacetate and sodium bisulfite. Spencer (59) also found inhibition of ethylene production by intact tomato fruit on introduction of 2,4 dinitrophenol. Since the dinitrophenol uncouples phosphorylation from respiration it is apparent that phosphorylation or a preformed source of energy is required for ethylene production. The data from experiments with apple plugs also indicate that sulfhydryl groups may be involved in biosynthesis of the volatile.

(ii) Substrates

Some early exploration with substrates included experiments in which apple plugs were soaked in radioactive solution for one hour (60). Administration of glycerol-1(2)(3)-^{14}C did not result in labeled ethylene, yet glycerol-treated tissue produced ethylene at 250 percent the rate of

water-soaked controls. Burg suggested that the boost in production arose from an osmotic effect of glycerol. Injection of uniformly labeled sucrose-^{14}C into the ovary of whole apples allowed the formation of radioactive ethylene, whereas acetate-1-^{14}C was less effective in this respect (60). Since no radioactive ethylene was obtained from metabolism of evenly labeled tyrosine-^{14}C by apple tissue slices, it was speculated that ethylene was not derived from aromatic compounds originating from sugars (60).

The 6-carbon of glucose was more effective than the other carbons in labeling ethylene in apple tissue slices (61). Experimentation with labeled substrates and Penicillium digitatum also demonstrated a higher specific activity of ethylene from metabolism of glucose-6-^{14}C than from glucose labeled in the other carbon positions (62). The results indicated that pyruvate underwent decarboxylation to acetyl-CoA before being incorporated into the ethylene molecule. A further suggestion was that ethylene originated from the middle carbon atoms of fumarate.

Some other experiments by Burg and Burg (63) with apple tissue slices incubated in medium containing pyruvate-2-^{14}C and acetate-1-^{14}C indicated decarboxylation of pyruvate and metabolism of acetate to ethylene. They speculated that carbons 1 and 2 of acetate formed ethylene as a unit since during the first 80 minutes after administration of the isotopes, carbons 2 and 3 of pyruvate and 1 and 2 of acetate were equally effective in forming ethylene. At the end of 200 minutes, however, the specific activity of ethylene arising from metabolism of acetate-2-^{14}C in their system was considerably higher than that from acetate-1-^{14}C. This in turn suggests that the carboxyl carbon of acetate is not always incorporated into the ethylene molecule. If ethylene arises from glucose through triose phosphate, carbon 6 of glucose becomes carbon-2 of acetate,

which in light of the above evidence would account for its relatively greater capacity to label ethylene. This does not, however, explain the nearly equal contributions of carbons 1 and 2 of glucose to the ethylene label (63). It was later proposed (64) that conversion of glucose to triose by both glycolysis and direct oxidation would account for the preferential incorporation into ethylene of carbons 5 and 6 relative to carbons 1 and 2 of glucose, since direct oxidation would utilize carbons 4, 5 and 6 exclusively.

Burg suggested the involvement of the tricarboxylic acid cycle in ethylene genesis (63), surmising as did Wang, Persyn and Krackov (62), that the volatile could have been derived from carbons 2 and 3 of fumarate. Such conjecture is not consistent with a recent publication (65) stating on the basis of rate experiments with labeled substrates that intermediates of the tricarboxylic acid cycle are, rather, converted to acetate through pyruvate and then proceed to ethylene by another pathway. Moreover, Phan-Chan-Ton (66) found that glucose served as a satisfactory substrate for ethylene and that malate did not appear to be a component of the direct synthetic route from glucose to ethylene.

Gibson (67) noted that substrates malate, citrate and pyruvate were better promoters of ethylene production by Penicillium digitatum than were glucose and mannitol. Wang and coworkers (68) on the basis of label studies with the same fungus suggested that ethylene is synthesized according to the following pathway:

$$\text{succinate} \longrightarrow \text{fumarate} \longrightarrow \text{malate} \longrightarrow \text{acrylate} \longrightarrow \text{ethylene} + CO_2$$

These results are in contrast to the data of Spalding and Liebermann (69) who found that 0.1 M glucose was more effective as a substrate for production of the volatile by Penicillium digitatum than 0.1 M malate,

pyruvate or citrate. Jacobsen and Wang (70) reported that in Penicillium digitatum, acrylate-2(3)-^{14}C was readily incorporated into the ethylene molecule, but that acrylate-1-^{14}C was not. This suggests a decarboxylation of acrylic acid to ethylene. Label studies with serine gave evidence for a high conversion efficiency of serine-3-^{14}C to ethylene by Penicillium digitatum (16). Such data ties in with the previously discussed (page 9) relatively high incorporation of acetate-2-^{14}C into the ethylene molecule.

(iii) Radioactive Ethylene

By application of ethylene-^{3}H and ethylene-^{14}C to intact green avocada fruit (2),(71), Jansen attempted to trace in reverse the pathway for ethylene biosynthesis. The data indicated that more tritium than carbon-14 was incorporated into toluene and benzene, suggesting that the hydrogen of ethylene is removed and metabolized by a different route from the carbon. Potential polymerization of the highly radioactive ethylene (40 mc per mmole) was avoided prior to experimentation by storage in dry ice, but thereafter this possibility remained; the exposures of avocados were of 4 hours duration.

(iv) Non-Enzymatic Models for In Vivo Production of Ethylene

A non-enzymatic system for ethylene production, composed of peroxidized linolenic acid reacting with cuprous ion in acetate buffer has been presented as a model system for in vivo production of the volatile in apple tissue (72). Ethylene production by apple tissue slices was inhibited by diethyldithiocarbamate but not by ethylenediaminetetracetic acid. The authors therefore suggested that cytoplasmic particles, lipoxidase and a copper enzyme may form a system for in vivo production of ethylene. Pertinent to this speculation though, is the fact that

dialkyldithio compounds are known to complex with other metal ions besides copper ion (73). It is interesting in view of this model system that lipoxidase activity in apple peel tissue was found to increase during the climacteric stage of development (42). Moreover, this increased activity corresponded quite closely to the ethylene rise characterictic of this developmental stage. Ethylene production has been found to be higher from the peel of apple than from the pulp tissue (42).

It is pertinent to these thoughts that some evidence disfavours linolenic acid as an ethylene precursor. Peroxidation of linolenate must occur prior to ethylene formation and it is questionable whether fatty acid peroxides exist in normal tissues in view of the susceptibility of enzymes to damage from peroxides (74). As well, if linolenic acid is a precursor of ethylene, production of the volatile by apple tissue slices is very inefficient relative to the high free linolenic acid levels present (74).

Ethylene was also formed in a model system involving a cuprous-catalyzed breakdown of methionine in the presence of hydrogen peroxide (75). Added methionine augmented ethylene production in apple tissue slices (76) and production was inhibited by diethyldithiocarbamate.

(v) Ethylene Production in relation to the Glyoxylate Pathway

Spencer and Olson (22) have reported that the biogenesis of ethylene does not appear to be promoted by functioning of the glyoxylate pathway. This is in agreement with the fact that ethylene production has been demonstrated in animal tissues (27), (28), in which the glyoxylate cycle is apparently inoperative.

(b) Pathway Studies with Subcellular Systems

(i) Subcellular Particles

Other studies on the biosynthesis of ethylene have been carried out using subcellular systems. Spencer (77) first reported that mitochondria from tomatoes produced ethylene. This discovery was later confirmed when it was found that mitochondria from rat liver, rat intestinal mucosa (27) and beef heart (28) also produced ethylene.

The use of subcellular particles, active in ethylene production, constituted a step forward in that the metabolic complexity of the system for research on the pathway was reduced. Inhibitor studies (78), (79), with a 35,000 g fraction, largely mitochondrial, from tomatoes included the effects of cyanide at 10^{-2} M and arsenite at 10^{-4} M. The former inhibited production of the volatile implicating the involvement of carboxyl or disulphide groups in ethylene biosynthesis. Slight inhibition by arsenite suggested that oxidative decarboxylation of keto acids was not directly involved. Iodoacetamide at 10^{-2} M and p-chloromercuribenzoate at 10^{-4} M decreased ethylene production. Glutathione at 10^{-2} M reversed the effect caused by p-chloromercuribenzoate, signifying a protective action on vulnerable sulfhydryl groups. Since monofluoroacetate at 10^{-3} M and malonate at 10^{-2} M did not significantly inhibit production of the volatile, the tricarboxylic acid cycle is probably not directly involved in ethylene production. Evolution of the volatile by the same fraction was considerably inhibited by cycloserine (79) implicating the involvement of transaminases.

Lieberman and Craft (80) prepared, from tomato and apple, subcellular particles that were apparently active in ethylene production in the presence of thiomalic and thioglycolic acids; the particles were isolated from acidic media. This work was later disputed however, since gas

chromatographic analyses indicated that the volatile from the acid particles was not ethylene (81).

Evidence has been reported (82) for the coupling of ethylene generation to reduced NADP and pyrophosphate-dependent peroxidation of lipids in the presence of cuprous ions in rat liver microsomes. This again suggests that in vivo production of the gas may be geared in part to the action of a copper-containing enzyme on such as linolenic acid, but pertinent to such a consideration are the previously discussed (page 12) arguments against linolenate as an ethylene precursor.

Substrate and cofactor studies were carried out on a subcellular fraction from tomatoes (79). Cofactors found to stimulate ethylene production were NADP, pyridoxal hydrochloride and thiamine pyrophosphate and magnesium ion. Substrates causing increased production of the volatile included ethanol, propionic acid, aspartic acid, glutamic acid, γ-amino butyric acid, β-alanine and serine. In chapter VI of this thesis the author will discuss these results in the light of those obtained with substrate studies on enzyme powders.

(ii) Enzyme Powder

Abeles and Rubinstein (24) have obtained cell-free ethylene evolution from etiolated pea seedlings. Their enzyme system was not prepared from a subcellular particulate fraction, but rather consisted of the supernatant from ammonium sulphate precipitation of a tissue homogenate that had been previously centrifuged at 12,000 g. Chandra and Spencer (39) have shown that the most active sites for ethylene production are the subcellular particles obtained at 35,000 g; although the fraction was composed of mitochondria primarily, it probably contained some microsomes as well.

The system of Abeles and Rubinstein (24) required a substrate consisting of the supernatant from an acetone precipitate of the original crude mixture. Cofactors ATP, biotin, CoASH, thiamine pyrophosphate, NAD, NADH, NADP, FMN and FAD had either no effect or inhibited production. Also, ethanol, ethionine, methionine, glycine, glycolic acid, ethane and acetyl-coenzyme A were added to the enzyme system in the presence of a limiting amount of crude substrate; none stimulated production of the volatile.

(c) β-alanine as a Precursor for Ethylene

Studies with a subcellular fraction from tomatoes indicated that β-alanine was a potential precursor for ethylene (79). It has been proposed that β-alanine is metabolized in Pseudomonas fluorescens by an initial transamination reaction (83). The amino acid has also been shown to be involved in propionic acid metabolism in Clostridium propionicum (84), animal tissue (85), and in higher plants (86). In view of the association of ethylene evolution with aging of tissue, it may be significant that β-alanine is a product of uracil catabolism and hence, possibly of nucleic acid breakdown, in a variety of tissues. For example, catabolism of uracil in rat liver slices occurs according to the following reaction sequence (87):

uracil ⟶ dihydrouracil ⟶ β-ureidopropionic acid ⟶ β-alanine

The same reaction sequence for degradation of pyrimidines apparently functions in Clostridium uracilium (88) and in parasitic flatworms (89). More recent studies have implicated the same mechanism for uracil catabolism in pine tissue (90) and rye seedlings (91). This conversion does then, appear to provide a source of β-alanine in many, if not all, tissues. More detailed work has indicated that in rat liver the reduction of uracil to dihydrouracil is the rate limiting step in catabolism of uracil to

β-alanine (92). It is interesting to speculate that with the onset of senescence and a corresponding boost in ethylene evolution, this enzymatic conversion would no longer remain rate limiting, thus favouring the formation of β-alanine and possibly ethylene.

At the outset of this investigation it was apparent that it would be advantageous to obtain a system as biochemically simple as possible, capable of catalyzing the formation of ethylene from added substrates. Considerable progress in this direction had been made with the use of the mitochondrion as a system for ethylene production. Consequently, the objective undertaken by the author was to prepare a partially purified enzyme powder from a subcellular fraction and thenceforth explore further the biosynthesis of ethylene. Prior to this though, since accelerated ethylene production by this subcellular fraction had previously been shown to be dependent upon initial particle intactness followed by controlled particle disintegration (93), various means for accomplishing such a breakdown were tried.

There are several advantages to undertaking metabolic studies with enzyme powders. Perhaps foremost is the fact that substrate experiments, especially those with labeled substrates, are less difficult as a result of the exclusion of many extraneous pathways. In addition, the objective of the isolation of radioactive intermediates is often more easily realized. Complications arising from penetration barriers when substrates and co-factors are added to tissue slices and even subcellular particles, are virtually eliminated by use of enzyme powders. Also, purification procedures allow a ready removal of endogenous substrate pools, making reaction sequence studies less burdensome. These advantages are certainly tempered however, by the fact that a purified system is more remote from its in vivo state.

Chapter II

MATERIALS AND METHODS

1. Procurement and Storage of Biological Tissues for Studies of Ethylene Biosynthesis

(a) Tomatoes

Tomatoes were used for studies of ethylene biosynthesis when they had developed to a stage near the climacteric peak in respiration, since ethylene evolution is known to be high at this period (31). Firmness and extent of colouring were the criteria followed in determining the readiness of the tomatoes for experimentation. Once ripened to the required degree, they were stored at -10°C and used within three weeks.

(b) Oat Seedlings and Bean Seedlings

Oats (Victory), *Avena sativa*, and beans (Kinghorn), *Phaseolus vulgaris*, were allowed to germinate at 24°C in the dark in previously sterilized glass trays of Perlite R covered with wet tissue, or in California soil mix. Immediately before planting, the seeds were soaked for 10 minutes in 50 percent sodium hypochlorite solution, washed in cold water, soaked for 5 minutes in 50 percent ethanol solution and then rewashed. The seedlings were harvested at 8 - 10 days after planting, since maximum ethylene evolution was found to occur at this stage during the development of oat seedlings (23). Once harvested, the seedling tissue was cut into small segments, stored at -10°C and used within 1 week.

(c) Bean Cotyledons

Many more seedlings were required when just the cotyledons were harvested. Consequently plants were grown in California soil mix or Vermiculite in large wooden flats in a growth chamber. The flats were

sterilized prior to planting, but it was not found necessary to treat the seeds nor sterilize the growth media, under these conditions. Cotyledons were harvested at 7 - 8 days after planting, since ethylene production was found to be maximal at this time, stored at -10°C, and used within 1 week.

2. Non-Biological Materials

All chemicals used throughout the investigation were reagent grade from either Fisher Scientific Co. Ltd., Eastman Organic Chemicals Department of Eastman Kodak Co., or Laboratory Chemicals Division of British Drug Houses Ltd. β-alanine-2-C^{14} was obtained from Volk Radiochemicals Co., MN-cellulose powder 300 and silica gel G from Canadian Laboratory Supplies Incorporated, and Cab-o-sil from Cabot Corporation, Boston, U.S.A.

3. Collections and Analyses of Ethylene

Ethylene was collected by bubbling purified air through the reaction vessel and thence through cold mercuric perchlorate solution (94) according to the method of Chandra and Spencer (29), (93).

For collections of the gas from intact wax bean cotyledons, 50 gm. quantities of cotyledons were placed in 1000 ml. Erlenmeyer flasks and in order to provide maximum circulation provision was made to pipe the incoming air to the bottom of the flask. Subcellular particles and enzyme powders with their respective reaction mixtures were placed in 50 ml. extraction flasks for ethylene collections. Continual stirring of the reaction mixtures throughout the collection periods promoted maximum transfer of ethylene to the air stream passing through the collection flasks into the mercuric perchlorate solution.

Quantitative analyses of ethylene were obtained by gas chromatography; the unit equipped with a flame ionization detector and constructed

by Chandra (93) was used. In some gas chromatographic analyses, however, 50 x 0.35 cm. dual columns of activated alumina coated with silicone (Burrell 341-116-02) were used instead of columns of squalane-coated firebrick. (The retention time for ethylene with the alumina column was 25.2 seconds.) The limit of sensitivity was 5 $m\mu l$ of ethylene per ml. of injected sample.

The identity of ethylene was established by a comparison of retention times of samples with those of ethylene standards. The presence of ethylene in the volatiles evolved from an enzyme powder was confirmed by mass spectrometry of a collection of these volatiles at dry ice - acetone temperature on silica gel (65).

4. Preparations of Subcellular Particulate Fractions from Tomatoes; Methods for Particle Disintegration

(a) Preparation

Subcellular particulate fractions from tomatoes were prepared by a method previously established in this laboratory (79), (93). Frozen tomatoes were cut up with a chisel and ground in a Waring blender at very low speeds (rheostat setting of 70 for 30 seconds) in ice-cold buffer containing 0.5 M sucrose and 0.5 M KH_2PO_4 adjusted to pH 8.0 with NaOH. (Earlier work in this laboratory had shown that careful grinding of the tissue was prerequisite to attaining a subcellular fraction active in ethylene production.) One thousand gm. of frozen tissue were used per litre of grind buffer. Subsequent to filtering through cheesecloth, starch and cell wall debris were removed from the tissue homogenate by centrifugation at 4080 g for 10 minutes at 0^oC with a Servall model RC-2 ultracentrifuge. The supernatant was filtered through broadcloth and re-centrifuged at 35,000 g for 15 minutes in a Beckman model L or L-2 ultra-

centrifuge at $0^{o}C$ yielding a pellet consisting primarily of mitochondria. This particulate fraction was suspended in ice-cold buffer containing 0.5 M sucrose and 0.125 M KH_2PO_4, previously adjusted to pH 7.0 with NaOH. (The pellet arising from a 35,000 g centrifugation of one litre of tomato "homogenate" was used per 20 mls. of buffer.) Suspension was accomplished by very slow stirring with a magmix at $0^{o}C$ and the suspended mixture was made to 40 or 60 ml. total volume with buffer depending on the need for either two or three 20 ml. samples for the particular experiment.

(b) Disintegration of Subcellular Particles

(i) By Sonication

For sonication the particulate suspension was transferred to the chamber of a Raytheon DF-101 10 kc. sonic oscillator and sonicated for 4 minutes at 1.2 amperes at $4^{o}C$; the temperature of the reaction mixture did not increase significantly during the treatment. For ethylene collection, the resulting solution was transferred to a reaction flask containing required substrates and cofactors. In the event of a second sonication, the entire reaction mixture was sonicated prior to the 22 - 23 hour ethylene collection period, under the same conditions as the initial treatment.

(ii) By Freezing and Thawing

To determine the effectiveness of fast freezing and subsequent thawing as a means of particle disintegration in relation to ethylene production, the particulate suspension was transferred to a reaction flask containing required cofactors and substrates, and frozen by complete submersion in liquid nitrogen. Thawing was effected by agitation in a $40^{o}C$

water bath and then ethylene collections were begun. The flask was stoppered throughout the entire procedure. A second treatment was carried out in an identical fashion prior to the 22 - 23 hour ethylene collection period.

(iii) Use of a Nossal Disintegrator

Mechanical fragmentation of mitochondrial suspensions by use of a Nossal disintegrator, and the subsequent isolation of particulate and soluble protein components required for oxidative phosphorylation, have been previously achieved (95). The Nossal unit can be purchased from McDonald Engineering Co., Ohio, U.S.A.

For treatment with a Nossal disintegrator in this investigation, 18 ml. of particulate suspension were added to a Nossal capsule (precooled to 0°C) along with 2 gm. of washed ground glass for abrasion; the abrasive was added slowly to the particulate suspension and subsequent gentle stirring allowed trapped air to escape. Prior to use, the ground glass was washed twice by shaking in the Nossal capsule (2 gm. of ground glass per 15 ml. of deionized water), rinsed thoroughly with 95 percent ethanol and finally dried at 120°C. This treatment minimized alkaline contamination from the glass during mitochondrial disintegrations. Possible denaturation by foaming was minimized by having the capsule brim full; considerable foaming was evident otherwise. The capsule was shaken for 0.5 minutes at 100 cycles per second and during this time, cooling was accomplished by spraying a stream of liquid carbon dioxide over the capsule.

5. Preparation of Enzyme Powders from Oat Seedlings, Wax Bean Seedlings and Wax Bean Cotyledons.

(a) Crude Enzyme Powders

For preparation of enzyme powders, frozen seedling tissue was ground for 15 seconds in ice-cold buffer containing 0.5 M sucrose and 0.2 M KH_2PO_4, adjusted to pH 7.0 with NaOH; the grinding was performed with a Waring blender at low speed (rheostat setting of 60 for 30 seconds). Sixty-six gm. of frozen tissue were used per 200 ml. of grind buffer. The ground mixture was strained through cheese cloth yielding a tissue homogenate. When only cotyledons were used, subsequent to freezing at -10°C they were crushed with a hydraulic press at 20,000 p.s.i. in ice-cold buffer of the above composition, and the resulting mixture strained. The tissue homogenates from both methods were initially centrifuged at 4080 g to get rid of starch and cellwall debris and then centrifuged again at 35,000 g yielding a subcellular particulate fraction, probably consisting primarily of mitochondria. Centrifugations were carried out in the same manner as for the tissue homogenate from tomatoes (page 19).

The pellet obtained from this centrifugation was suspended in cold deionized water to allow some osmotic disruption, and then three consecutive times was frozen in dry ice-acetone and thawed in a 40°C water bath. (The pellet arising from centrifugation at 35,000 g of one litre of tissue "homogenate" was suspended in 10 ml. of deionized water. In experiments where the freeze-thaw treatment was replaced by a more extensive osmotic treatment, the pellet was suspended in 100 ml. of deionized water and allowed to disrupt for 1 hour.)

This suspension was then subjected to ammonium sulphate fractional precipitation. Cold saturated ammonium sulphate solution, brought to pH 7.0 with sodium hydroxide, was used. The fraction that separated after

1.5 hours at 5°C at 66 percent saturation with ammonium sulphate was spun down at 18,800 g for 10 minutes at 0°C in a Servall model RC-2 ultracentrifuge and subsequently lyophilized. This powder constituted a crude enzyme system that, in the presence of appropriate substrates and cofactors was active in ethylene production.

(b) Partially Purified Enzyme Powders

For purification, lipids were removed from the crude powder by a butanol extraction. To achieve this, the powder was suspended in cold absolute n-butanol (50 ml. per gm. of powder) by use of a Servall Omnimixer at low speed (rheostat setting of 30) for 30 minutes; the suspension was centrifuged at 18,800 g for 10 minutes at 0°C in a Servall model RC-2 ultracentrifuge and the supernatant discarded. The residue was again treated with butanol and centrifuged as above. The precipitate was partially dried on filter paper under suction, in an atmosphere of nitrogen.

Further purification was obtained by intensive ammonium sulphate fractionation. The material on the filter paper was suspended in cold deionized water (10 ml. water per gm. of initial crude powder) for 5 minutes before addition of ammonium sulphate. The suspension was fractionated into 0 - 20, 20 - 65, and 65 - 100 percent saturated ammonium sulphate fractions. All ammonium sulphate precipitates were allowed to aggregate at 5°C and were subsequently spun down at 18,800 g for 10 minutes at 0°C in a Servall model RC-2 ultracentrifuge. For preparation of the 65 - 100 percent fraction the supernatant from the 20 - 65 percent fraction was dialyzed against deionized water for 24 hours at 5°C and then lyophilized. Since both the 20 - 65 and 65 - 100 percent fractions were active in ethylene production, in most experiments a 35 - 100 per-

cent saturated ammonium sulfate fraction was used in order to incorporate a large portion of the activity. To obtain this fraction, the supernatant from the 0 - 20 percent fraction was dialyzed against 6 litres of deionized water for 24 hours at 3°C with three water changes at intervals of eight hours and finally lyophilized, yielding a partially purified enzyme powder active in ethylene production.

6. Preparation of the Reaction Mixture for Chromatography

After 24 hour ethylene collection periods, the reaction mixtures containing enzyme powder, substrates and cofactors, were prepared for paper chromatography and thin layer chromatography in the following manner. Ethylamine salts of acids in the mixture were formed by adjusting the pH to 10 or above with 33 percent (v/v) ethylamine solution in deionized water. Concentration to semi-dryness was accomplished in a Buchler Rotary Evapo-mix at 40°C; the reaction mixtures were diluted to 4 ml. with deionized water, made basic to pH 10 or above with 33 percent ethylamine solution and brought to a final volume of 4.5 ml. with deionized water. The concentrated samples were stored at -10°C.

Immediately prior to spotting, it was necessary to centrifuge the concentrated solutions to rid them of sugars and polysaccharides that interfered with chromatogram development. (An International model HN centrifuge was used at full throttle for 5 minutes at 25°C.) All spots were dried with a cool air stream.

The proposed intermediates propiolactone, acrylic acid and acetic acid were not produced in sufficient concentration for detection by indicator spray when separated by thin layer chromatography. Where these substances had not been included as part of the reaction mixture for

ethylene production, known amounts of their ethylamine salts were added as markers prior to concentration of the mixture for analysis. Final concentrations of 70 mM in the 4.5 ml. were used consistently and this amount was sufficient for location on the chromatograms by indicator spray.

7. Paper and Thin Layer Chromatography of the Reaction Mixture

For paper chromatography 100 λ of the reaction mixture was spotted on Whatman No. 1 filter paper (46 cm. x 57 cm.) and two solvent systems were used. Acrylate, acetate and propiolactone were separated by development with water-saturated n-butanol (96) in a tank that was previously equilibrated with 0.125 percent ethylamine in n-butanol-saturated water; for good separation, it was not necessary to equilibrate the paper. β-alanine, malonate and α-ketoglutarate were separated by development with 20 percent NH_4OH in 95 percent ethanol; no equilibration was necessary.

For thin layer chromatography, 0.25 mm. layers of either MN-cellulose powder 300 (20 percent in deionized water) or silica gel G (33 percent in deionized water) were spread on glass plates (20 cm. x 20 cm.) with a Desaga thin layer chromatography apparatus. After drying the plates were activated at 110°C under vacuum for 1 hour. Then 5 λ and 10 λ spots of the reaction mixture were resolved by three different systems. Acrylate, acetate and propiolactone were resolved on cellulose-layered thin layer chromatography plates and β-alanine, malonate and α-ketoglutarate on silica gel G plates, although cellulose plates were sometimes used as well for the latter group. The solvent systems used in both cases were the same as employed for paper chromatography. Equilibration of the tank, but not the plate, was required for the cellulose system and no equilibration was required for the silica gel system. Since β-

hydroxypropionate was masked by β-alanine, α-ketoglutarate and malonate on cellulose plates developed in water-saturated n-butanol, it was separated from all the other acids on silica gel G plates with a solvent system composed of 20 percent NH_4OH in methanol; no equilibration was required. This system also gave improved resolution of α-ketoglutarate from β-alanine and malonate.

For both paper chromatography and thin layer chromatography, the chromatograms were allowed to develop to almost their entire length, with occasional exceptions when paper chromatograms were run off the end for better resolution. Detection was accomplished in both types of chromatography by spraying the n-butanol-developed chromatograms with 0.4 percent chlorophenol red in 95 percent ethanol, and the NH_4OH-ethanol and NH_4OH-methanol-developed chromatograms with 0.4 percent chlorophenol red in 95 percent ethanol, made basic to pH 10 or more with dilute NaOH.

Thin layer chromatographic data were confirmed by developing the chromatograms in a second direction. Chromatograms that had initially been developed in the n-butanol solvent or the NH_4OH-methanol solvent were redeveloped in a second direction in isopropanol-NH_4OH - H_2O in the volumetric ratio of 8:1:1; those initially developed in the NH_4OH-ethanol solvent were redeveloped in n-propanol-methanol-NH_4OH-H_2O in the volumetric ratio of 4:4:1:1. In both instances of 2 dimensional chromatography, the chromatograms were sprayed with the basic chlorophenol detection solution.

8. Determination of the Radioactivity of Ethylene

When ^{14}C-labelled ethylene was used, it was collected in mercuric perchlorate and the radioactivity determined with a Nuclear Chicago liquid scintillation unit, Model 725, operated at 5°C. Mercuric perchlorate,

containing the labelled gas as a mercury-ethylene complex, was mixed with anthracene and Tween 81 in the ratio 3g anthracene:2 ml. perchlorate: 1 drop of Tween 81. The mixture was stirred to a dry paste and counted. Of the original 4.5 mls. of mercuric perchlorate in which ethylene was collected, 2 mls. were used for quantitative analysis of ethylene by gas chromatography and 2 mls. for the radioactivity determination.

9. Determination of Radioactivities and Specific Activities of the Intermediates

Paper chromatograms of reaction mixtures containing labeled components were scanned with a Labitron Monitor, model 1619A, fitted with a Nuclear Chicago recorder, model R1000A. For radioactivity determinations, spots were scraped from thin layer chromatography plates into liquid scintillation counting vials containing 0.5 gm. of Cab-o-sil (97). Fifteen ml. of a mixture 0.4 percent in 2,5-diphenyloxazole and 0.005 percent in 2,2^1-paraphenylene bis-5 phenyloxazole, in toluene, were added to each vial; the vials were shaken to suspend the labeled particles in the Cab-o-sil and counting was done with a Nuclear Chicago liquid scintillation unit, model 725, operated at 5°C and sometimes at room temperature.

For determinations of specific activities of acetate and malonate, their concentrations were ascertained spectrophotometrically. Ethylamine salts of the acids were isolated by thin layer chromatography of 10 λ aliquots of the concentrated reaction mixtures. The spots were scraped from sprayed thin layer chromatography plates and the acids eluted by shaking with 4.5 ml. of deionized water for one hour. These suspensions were cleared by centrifugation with an International model HN centrifuge at full throttle for 2 minutes at 25°C. Four ml. aliquots of the solutions

of the acids were made to pH 3 with dilute hydrochloric acid. The solutions were subsequently quantitatively transferred to 25 ml. volumetric flasks containing 5 ml. of a 1:1 mixture of 1.25×10^{-3} M benzidine hydrochloride (1 ml. of 30 percent acetic acid was required in each 100 ml. of this solution) and 3.75×10^{-3} M copper acetate (98). The solutions were made to volume with deionized water prior to reading. Spots serving as blanks were scraped from the thin layer chromatography plates and treated in the same manner. Standard curves were prepared from solutions of the ethylamine salts of the two acids. Absorbancies were read at 242 mμ (98) on a Beckman D.U. spectrophotometer.

10. Determination of Malonic Acid Levels in Wax Bean Cotyledons

(a) Preparation of a Tissue Extract

Wax bean cotyledons were harvested at daily intervals from 4 to 9 days after planting. The cotyledons were frozen at -10°C and lyophilized. Extracts were prepared from the daily quantities of lyophilized tissue in the following manner. Fifty gm. of dry cotyledon tissue were ground to a fine powder with a mortar and pestle and extracted with 250 ml. of boiling 95 percent ethanol for 15 minutes; stirring during extraction was accomplished with a Servall Omnimixer at low speeds (rheostat setting of 20 - 30). The supernatant was decanted and the residue extracted again in the same fashion. The suspension from the second extraction was strained through cheese cloth and the supernatants from both extractions were filtered under suction through #2 MM filter paper and evaporated to dryness under vacuum at 40°C with a Buchler flash evaporator.

The residue was dissolved in 100 ml. of 65 percent ethanol, adjusted to pH 10 with 50 percent NH_4OH and extracted with 500 mls. of

chloroform (99). After discarding the chloroform extract, the remaining aqueous layer was acidified with 2N hydrochloric acid and evaporated to dryness with a flash evaporator under the same conditions as above. The residue was dissolved in 4.5 ml. of deionized water and a 4 ml. aliquot, following dilution to 15 ml., was centrifuged at 59,000 g for 15 minutes in a Beckman model L-2 ultracentrifuge. The supernatant was analyzed for malonic acid content.

Throughout the preparation of this extract all transfers were as quantitative as possible.

(b) Isolation and Quantitative Determination of Malonic Acid

Malonic acid was isolated from the tissue extract by thin layer chromatography. For this purpose 1 ml. of supernatant was diluted to 3 ml. total with deionized water and made 14 mM with malonic acid; malonate was added as a marker for chromatography. Aliquots of 5 λ were spotted on cellulose layered plates and developed with a solvent of chloroform-ethanol (95 percent)-formic acid (90 percent) in the volumetric ratio of 50:50:1 (100). The plates were dried in a fume cabinet overnight and then heated at 110°C for 30 minutes to allow complete evaporation of formic acid. Detection was accomplished by spraying with 0.4 percent bromcresol green in 95 percent ethanol, made basic with dilute NaOH.

Quantitative determinations of malonic acid by spectrophotometry were carried out in the same manner as for determinations of specific activities (page 27) except that a standard curve was prepared from a solution of malonic acid rather than its ethylamine salt. In addition, it was not necessary to adjust the pH of the eluted solutions, since the acid was not in its salt form.

11. Snell Reaction

For the Snell reaction, β-alanine (1.0 M), pyridoxal hydrochloride (0.0325 M) and Cu^{2+} (0.625 mM, added as $CuSO_4$) were prepared in 0.2 M acetate buffer of pH 4.5, and mixed in the volumetric ratio of 2:2:1 in glass vials sealed with serum stoppers. The vials were autoclaved (101) at 20 lb. steam pressure for 30 minutes, with fast exhaust, and allowed to cool immediately.

Ethylene was quantitatively determined by gas chromatography. For this analysis the volatiles from the autoclaved vials were exhaustively transferred by a gastight syringe to 6.4 ml. serum vials containing 4.0 ml. of mercuric perchlorate (0.25 M in 2.0 M $HClO_4$), stoppered, and shaken for 1 hour at 0°C.to allow complexing of olefins with the mercury (94). Other volatiles were then removed from the space above the solution by a stream of ethylene-free air, and the ethylene was liberated and analyzed according to the methods of Chandra and Spencer (29), (93).

The ethylene was further identified by mass spectrometry. For these determinations 20 ml. of each of 1.0 M β-alanine and 0.0325 M pyridoxal hydrochloride, and 10.0 ml. of 0.625 mM Cu^{2+}, all in 0.2 M acetate buffer at pH 4.5, were combined in 60 ml. break-seal glass flasks. The components in the reaction vessel were frozen in dry ice-acetone and the vessel was evacuated to 1 mm. Hg. This vacuum was maintained during slow thawing, and the flask was then sealed before being autoclaved. Such a procedure maintained a gaseous volume and pressure optimal for injection of the complete gaseous sample into the mass spectrometer for analysis.

The progress of the reaction was confirmed by quantitative determination (102) of pyridoxal hydrochloride before and after autoclaving.

For this purpose, 0.1 ml. of the mixture from the Snell reaction was diluted to 10.0 ml. with 0.2 M acetate buffer, pH 4.5, and a 2.0 ml. aliquot was treated with 2.0 ml. of 50 percent ethanolamine solution. The absorbancy of the colored complex, presumably a Schiff's base formed between pyridoxal and ethanolamine, was determined on a Beckman model D.U. spectrophotometer at 365 mμ. A standard curve was prepared for each system since solution components affected the absorption at this wave length.

Chapter III

ASSOCIATION OF ETHYLENE EVOLUTION WITH AGING

1. Ethylene Production by Senescent Wax Bean Cotyledons

Cotyledons from Kinghorn wax beans (*Phaseolus vulgaris*), served as the tissue source for many of the enzyme preparations used in this investigation. The cotyledons were harvested when senescent (7 to 8 days after planting), since their ethylene production was at a peak at this time (Fig. 1); the tissue was withered and showed distinct signs of aging. At senescence mitochondria might be expected to undergo a decrease in activity and number (103), and although it is difficult to know definitely if mitochondrial disintegration occurs, it may be significant that *in vitro* production of ethylene has been shown to be associated with mitochondrial disruption (39). Opik recently reported (104) that in intact cotyledons of *Phaseolus vulgaris*, mitochondrial activity fell from 36 hours after planting, onwards. In the storage cells, by the third day of germination the cristae of the mitochondria had become swollen and the matrix had darkened. Although oxygen uptake reached a peak between the third and fifth days of germination, he attributed the continuing rise in respiration rate beyond 36 hours to an increase in soluble oxidative activity.

Accelerated ethylene evolution by senescent wax bean cotyledons is complementary to the fact that increased production of the gas is known to accompany senescence of other tissues (31), (34) and further indicates a connection between ethylene evolution and aging.

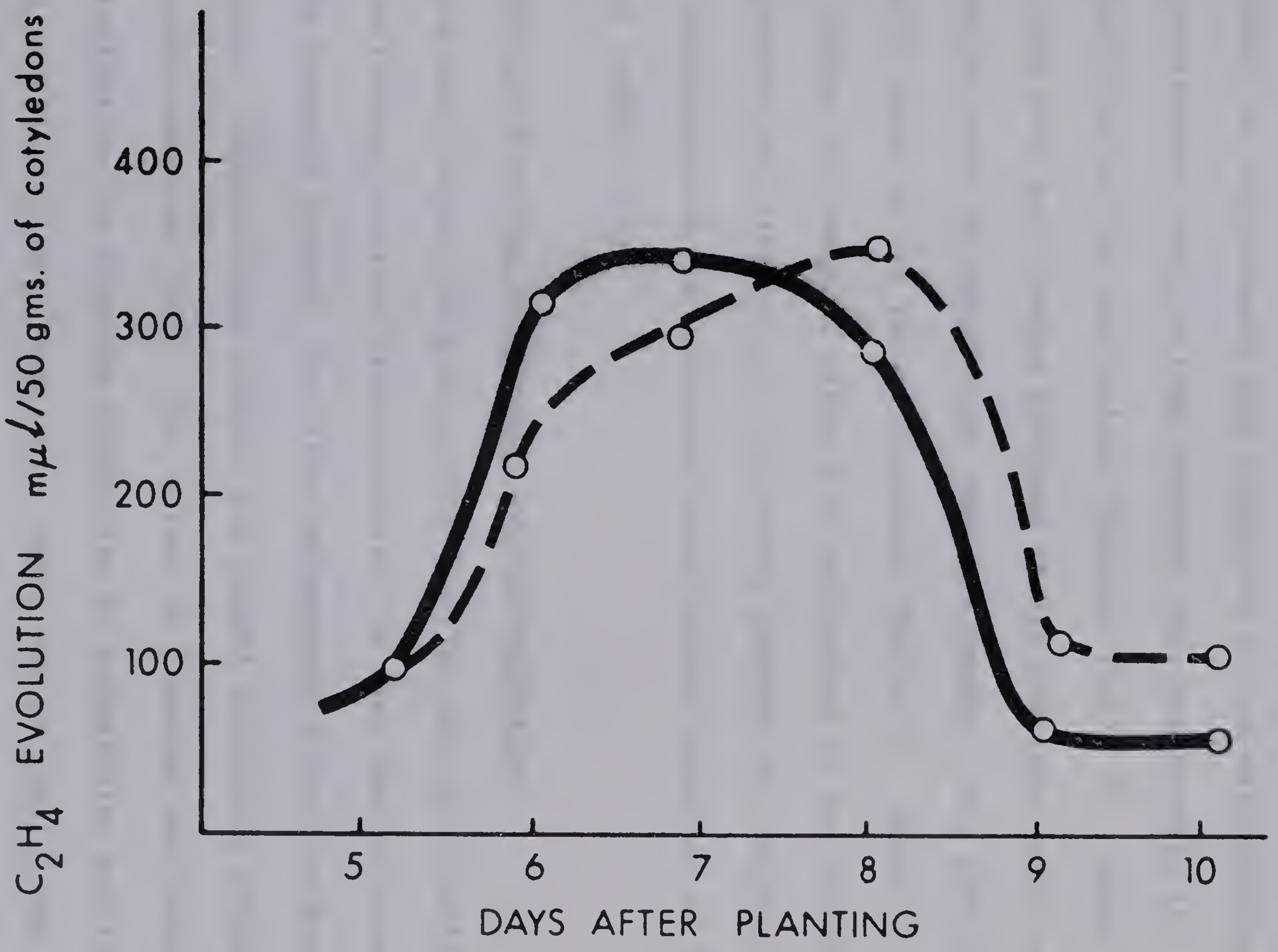


Fig. I. Ethylene Production by Bean Cotyledons: 0 - 2 hr. after harvest (———); 2 - 4 hr. after harvest (-----)

2. Ethylene Production by a Particulate Fraction; An Evaluation of Methods for Particle Disruption

In order to prepare enzyme powders active in ethylene production from a subcellular particulate fraction, it was necessary to disrupt the particles and release enzymes responsible for formation of the volatile. It is significant in this respect that evolution of the volatile from a subcellular particulate fraction from tomatoes was considerably increased by sonication of the particulate fraction (Table I). Intact mitochondria that were not treated produced little ethylene until considerable reaction time had progressed, when presumably, they partially disintegrated by aging in the reaction mixture (Table I). Since it is difficult to assess the scope of effects of sonication of the fraction on ethylene production, alternative less severe methods for accomplishing particulate disintegration and consequent accelerated evolution of the volatile, were sought (137).

(a) Effects of Freezing and Thawing versus Sonication

Freezing and thawing the particulate fraction was one potential means of accomplishing particle disintegration, in that disruption would be mediated by shearing forces. Rat liver mitochondria that had been frozen and thawed, released some enzymes and showed structural changes such as disrupted membranes (105). The effect of freezing and thawing, relative to sonication, on ethylene production by subcellular particles from tomatoes is illustrated in Table II. There was no effect from the initial freeze-thaw treatment, but the second treatment did boost ethylene production (Table II). In addition the total yield of ethylene from the sample treated by freezing and thawing was approximately equal to that of the sonicated sample. It is difficult to draw a definite conclusion

TABLE I

Effect of Sonication on Ethylene Production by a Subcellular Particulate Fraction from Tomatoes

Collection period hours	Ethylene, mμl/mgm of nitrogen	
	Untreated	Treated
0 - 1	N	14
1 - 3	5	2
3 - 22	40	N
22 - 23	T	79
23 - 24	15	48
Total	60	143
mgm of nitrogen	7.50	7.66

N = not detectable (less than 5 mμl); T = trace amount.

The two samples were from different preparations of mitochondria.

Sonications were performed on the treated sample prior to the 0 - 1 hour and 22 - 23 hour collection periods.

Reaction mixture: both samples contained mitochondria suspended in 20 ml. of buffer (0.5 M sucrose and 0.125 M phosphate, pH 7.0), and were made 1.5 mM in ATP after the initial treatment on one.

TABLE II

Effects of Sonication versus Freezing and Thawing on Ethylene Production by a Subcellular Particulate Fraction from Tomatoes

Collection period hours	Ethylene, mμl per mgm. of nitrogen Sonicated	Frozen and Thawed
0 - 1	10	N
1 - 3	2	N
3 - 21	13	26
21 - 22	N	22
22 - 23	75	30
23 - 24	59	88
Total	159	166
mgm. of nitrogen	7.45	7.0

N = not detectable (less than 5 mμl).

Treatments of freezing and thawing, and sonication, were performed prior to the 0 - 1 hour and 22 - 23 hour collection periods.

Freezing was in liquid nitrogen and thawing at 40°C.

Reaction mixture: both samples contained mitochondria suspended in 20 ml. of buffer (0.5 M sucrose and 0.125 M phosphate, pH 7.0), and were made 1.5 mM in ATP after the initial treatments.

regarding the capacity of such a treatment to activate the ethylene-producing system though, since the increased production may only reflect a release of ethylene from solution, caused by freezing (Appendix, Page 89). (Intact mitochondria were found to evolve ethylene when aged in buffer and ATP overnight (Table I).

Although the tomatoes were initially stored at -10°C (Chapter II), subcellular particles from frozen tomatoes and those from fresh tomatoes were found to produce ethylene in a similar fashion (79). In addition, it is known that other types of tissue can withstand freezing. For example, frozen tumors stored at -20°C maintained their transmissibility for up to 3 weeks (106).

(b) Effect of Ribonuclease

Addition of ribonuclease at 0.9 mgm. per ml. of particulate suspension had no effect on ethylene production by a subcellular fraction from tomatoes. This is not surprising in view of the fact that the enzyme would not be expected to attack main structural components such as lipo-proteins.

(c) Effect of Nossal Disintegration

Treatment of the subcellular fraction with a Nossal disintegrator had little effect on the overall production of ethylene relative to a control (Table III) and hence was of no potential use for enzyme powder preparations. Buffering action was sufficient to eliminate any changes in pH during the Nossal shaking, as long as the glass beads were initially treated as described in Chapter II.

TABLE III

Effect of Nossal Disintegration on Ethylene Production
by a Subcellular Particulate Fraction from Tomatoes

Collection period hours	Ethylene, mμl per mgm. nitrogen Untreated	Treated
0 - 1	17	N
1 - 3	4	21
3 - 20	93	56
20 - 21	3	6
21 - 24	10	25
Total	127	108
mgm. of nitrogen	6.56	6.07

N = not detectable (less than 5 mμl).

Nossal disintegrations were performed on the treated sample prior to the 0 - 1 hour and the 20 - 21 hour collection periods.

Reaction mixture: both samples contained mitochondria suspended in 25 ml. of buffer (0.5 M sucrose and 0.125 M phosphate, pH 7.0) and were made 1.5 mM in ATP after the initial Nossal treatment.

Chapter IV

PREPARATION OF ENZYME POWDERS

1. Tissue Grinding and Crushing

The method used for tissue grinding was found to be very critical. If the treatment with the blender was too severe, the ethylene-producing activity of the enzyme powders was greatly reduced and sometimes completely lost. Crushing the tissue hydraulically, as was done with the frozen cotyledons, was found to be a less drastic treatment, giving a greater yield of structurally intact mitochondria than did the grinding technique. Other experiments (49) were performed, in which the intactness of mitochondria isolated by this method was indicated by the presence of respiratory control in response to added substrates and cofactors.

Of the methods for particle disintegration attempted (Chapter III), the treatment by freezing and thawing was the only one, besides sonication, with any effect. Since some ethylene is produced by simple aging of mitochondria in a reaction mixture, it was thought that disruption entirely by an osmotic effect might suffice as a means of releasing enzymes from the particles for preparation of powders. Ethylene production by enzyme powder prepared from particles disrupted in this manner was lower, however, than that by powder prepared from particles disrupted primarily by freezing and thawing. In view of this and the necessity, in instances of an osmotic treatment only, for centrifugation of larger volumes to obtain the ammonium sulphate precipitate from the disintegrated particles, the freeze-thaw treatment was chosen for particle disruption.

2. Ammonium Sulphate Precipitations from Disrupted Mitochondrial Suspensions

Mitochondrial suspensions were frozen and thawed, as described previously (Chapter II), for particle disruption and subsequently sugjected to ammonium sulphate precipitation; an additional effect of the added salt may have been partial stabilization of proteins after disruption of membranes. A 50 percent saturated ammonium sulphate precipitation from the particulate suspension left approximately 20 percent of the total ethylene-producing activity in the supernatant layer (Table IV). In subsequent preparations, in order to incorporate as much of this activity into the powders as possible, a 66 percent saturated ammonium sulphate precipitate was taken from the disrupted particulate suspension. (At salt concentrations higher than the latter level, as a result of the high density of the supernatant it was not possible to obtain a compact pellet when spinning out the precipitate by centrifugation.) Subsequent lyophilization of the "66 percent precipitate" allowed drying with a minimum of denaturation.

3. Removal of Lipid Material by Extraction with Absolute n-Butanol

Some enzymes are denatured when exposed to organic solvents such as n-butanol in the presence of water. These same enzymes, however, are frequently moderately stable when exposed to dry organic solvents (107).

The enzymes involved in the biosynthesis of ethylene apparently behave in the manner described above. When the crude enzyme powder was extracted with n-butanol in the presence of water, ethylene-producing activity was completely lost; extraction of the dry powder with absolute n-butanol yielded a residue active in ethylene production (Table V).

Prior to extraction, the powder was dispersed as finely as possible

TABLE IV

Ethylene-producing Activity of a Crude Enzyme Powder and the Corresponding Supernatant Layer, from Oat Seedlings

Collection period hours	Treatment	Ethylene, mμl	
		Supernatant	Powder
0 - 2	none	7	45
2 - 20	none	7	17
20 - 21	none	20	68
21 - 23	frozen and thawed	N	N
Total		34	130

N = not detectable (less than 5 mμl).

The enzyme powder was prepared from a 50 percent saturated ammonium sulphate precipitate from a disrupted particulate suspension.

The volume of supernatant layer used was that from which 0.5 gms. of enzyme powder (the weight of powder used) had been precipitated.

Reaction mixture: 20 ml. of buffer (0.5 M sucrose and 0.125 M phosphate, pH 7.0), 50 mM β-alanine, 1.5 mM ATP, 0.5 mM pyridoxal phosphate, 0.2 mM Fe^{3+} added as $Fe_2(SO_4)_3$.

TABLE V

Denaturation by Water during n-Butanol Extraction of Crude Enzyme Powder from Wax Bean Cotyledons

		Ethylene, mμl	
Collection period, hours	Treatment	Butanol extraction of dry powder	Butanol extraction of powder suspended in substrate buffer
0 - 1	none	11	N
2 - 20	none	62	N
20 - 21	none	N	N
21 - 23	frozen and thawed	68	N
Total		141	N
Powder: mgm./ml.		25	25

N - not detectable (less than 5 mμl).

The samples were from different batches of comparably active crude enzyme powders.

Reaction mixture: buffer (0.5 M sucrose and 0.125 M phosphate, pH 7.0), 50 mM β-alanine, 1.5 mM ATP, 2.0 mM TPP, 1.0 mM Mg^{2+} added as MgSO$_4$.

with a mortar and pestle to allow rapid penetration of butanol into the particles. In addition to extracting lipids, the organic solvent probably served to partially disrupt lipo-protein complexes. Extractions were performed in a dry ice-acetone bath and with very slow stirring to minimize denaturation.

4. Ammonium Sulphate Fractionation of the Butanol-Treated Enzyme Powder

Further purification of the enzyme system was achieved by ammonium sulphate fractionation of the butanol-treated residue. The results of such a fractionation are illustrated in Table VI. Precipitation at 0 - 20 percent saturation with ammonium sulphate yielded a fraction inactive in ethylene production; this fraction probably consisted primarily of insoluble material. The 20 - 65 percent fraction was active in ethylene production and the 65 - 100 percent fraction was approximately 6.8 times more active. In the majority of preparations, a 35 - 100 percent fraction was used in order to incorporate a large portion of the activity.

Comparing the activities per mgm. of powder of the fractions with that of the crude powder in Table VI, substantially increased activity is apparent with each step in purification, although it is important to bear in mind that the crude powder and the fractions are from different batches of enzyme powder. Data in Table VII however, illustrate the relative ethylene-producing activities of crude and partially purified powders from the same batch preparation. In this experiment, extra cofactors were added to the purified system to compensate for losses during dialysis. There is about an eleven-fold increase in activity of the purified system relative to the crude (Table VII).

TABLE VI

Ethylene-producing Activity of Crude and Partially Purified Enzyme Powders from Wax Bean Cotyledons

Collection period, hours	Treatment of Suspension	Ethylene, mµl			
		Crude Powder	0 - 20 percent saturation with ammonium sulphate	20 - 65 percent saturation with ammonium sulphate	65 - 100 percent saturation with ammonium sulphate
0 - 2	none	28	N	62	28
2 - 20	none	141	N	124	203
20 - 21	none	17	N	28	N
21 - 24	frozen and thawed	34	N	56	124
Total		220	N	270	355
Powder: mgm./ml.		25	22.6	9.6	1.8
Total/mgm./ml.		8.8	N	28.1	197

Crude powder was from a different batch preparation than the fractions.

N = not detectable (less than 5 mµl).

Reaction mixtures: all samples contained buffer (0.5 M sucrose and 0.125 M phosphate, pH 7.0) and were 50 mM in β-alanine, 1.5 mM in ATP, 2.0 mM in TPP, 1.0 mM in Mg^{2+} added as $MgSO_4$, and 0.17 mM in CoA. The 65 - 100 percent fraction contained 0.5 mM pyridoxal phosphate and 0.2 mM Fe^{3+} added as $Fe_2(SO_4)_3$. (Other experiments have shown that the 0 - 20 percent fraction is inactive on addition of pyridoxal phosphate and Fe^{3+}.)

TABLE VII

Relative Activities of Crude and Partially Purified Enzyme Powders from Wax Bean Cotyledons

Collection period hours	Treatment	Ethylene, $m\mu l$	
		Crude	Purified
0 - 2	none	100	45
2 - 20	none	236	39
20 - 21	none	146	N
21 - 24	frozen and thawed	62	100
Total		544	184
Powder: mgm./ml.		25	0.75
Total/mgm./ml.		22	245

Both samples were from the same batch of enzyme powder.

N = not detectable (less than 5 $m\mu l$).

Reaction mixture: both samples contained buffer (0.5 M sucrose and 0.125 M phosphate, pH 7.0), and were 50 mM in β-alanine, 1.5 mM in ATP, 2.0 mM in TPP and 1.0 mM in Mg^{2+} added as $MgSO_4$. In addition the purified sample was 0.5 mM in pyridoxal phosphate, 0.2 mM in Fe^{3+} added as $Fe_2(SO_4)_3$, 50 mM in α-ketoglutarate and 0.17 mM in CoA.

Chapter V

PROPERTIES OF THE ENZYME POWDERS

1. Homogeneity of Individual Enzyme Powders

The degree of homogeneity of both crude and partially purified enzyme powders within the same batch preparation, is indicated in Table VIII. Variation in ethylene production from equal weights was approximately 4.2 percent for crude powder and 2.3 percent for partially purified powder. (Recovery of ethylene was consistently 100 percent for the collection method used (93).) Representative nitrogen values for the powders are given in the appendix.

There was some variation in levels of ethylene obtained from sample to sample during specific collection periods (Table VIII), since stirring of the reaction mixtures during ethylene collection was not entirely reproducible. Freezing and thawing of the reaction mixtures prior to the last ethylene collection, however, did release ethylene still in aqueous solution.

Enzyme activity of different batch preparations was not reproducible. This was to be expected in view of such difficulties as controlling variations in different batches of tissue grown at different times; in wax bean cotyledons, ethylene production is high within a relatively narrow range of development and biochemical changes in the tissue are probably quite rapid at this stage. As well, although attempts were made to prevent it, the degree of damage incurred by the enzyme system during the preparative procedures of grinding or crushing and lipid extraction probably varied among preparations.

TABLE VIII

Activities of Duplicate Samples of Crude and Partially Purified Enzyme Powders

Collection period, hours	Treatment	Ethylene, mμl			
		Crude		Partially purified	
0 - 2	none	56	56	N	N
2 - 20	none	101	85	23	23
20 - 21	none	45	51	56	51
21 - 24	frozen and thawed	62	61	96	105
Total		264	253	175	179
Powder: mgm./ml		25		1.5	

N = not detectable (less than 5 mμl).

The crude powder was from oat seedlings and the partially purified powder from wax bean cotyledons.

Reaction mixture: all samples contained buffer (0.5 M sucrose and 0.125 M phosphate, pH 7.0) and were 1.5 mM in ATP. The partially purified samples were in addition 50 mM in β-alanine, 2.0 mM in TPP, 1.0 mM in Mg^{2+} added as $MgSO_4$, 0.5 mM in pyridoxal phosphate, 0.2 mM in Fe^{3+} added as $Fe_2(SO_4)_3$, 50 mM in α-ketoglutarate and 0.17 mM in CoA.

2. Sensitivity of the Enzyme Powders to pH Changes and Storage

The *in vitro* enzyme system for ethylene production was quite sensitive to small changes in pH, being virtually inactive outside the range 6.7 - 7.2. Furthermore, the ethylene-producing activity of the enzyme preparation decreased markedly with time, and 2 weeks storage under vacuum in a desiccator at 5°C rendered the powders much less active (Table IX).

3. Substrate and Cofactor Studies with the Enzyme Powders

(a) Adenosine Triphosphate

The requirement by the enzyme powders of an energy source for ethylene production is illustrated in Table X. Both the crude and partially purified enzyme systems are inactive in the absence of added ATP. The 15 mµl of ethylene produced by the partially purified system in the absence of ATP (Table X, Sample 2A), may reflect a release of preformed ethylene that remained enzymatically bound throughout the preparative procedures. A more complete substrate and cofactor complement was added to the partially purified system to compensate for losses by dialysis. In some later experiments, ATP was used at 3 mM rather than 1.5 mM since the higher concentration caused a greater stimulation of ethylene production. A reaction mixture of the entire substrate-cofactor complement in buffer in the absence of enzyme powder yielded no ethylene.

Increased activity in the presence of ATP and no activity in its absence are consistent with enzymatic behaviour and may serve to indicate that at least some of the reactions catalyzed by this system are enzymatic.

TABLE IX

Effect of Storage under Vacuum in a Desiccator at 5°C on the Ethylene-producing Activity of a Crude Enzyme Powder from Wax Bean Cotyledons

Collection period hours	Treatment	Ethylene, mμl Fresh	Ethylene, mμl Aged by 2 weeks
0 - 2	none	56	79
2 - 20	none	106	N
20 - 21	none	51	N
21 - 24	frozen and thawed	112	73
Total		325	152
Powder: mgm./ml		25	25

N = not detectable (less than 5 mμl).

Samples were from the same batch of enzyme powder.

Reaction mixture: both samples contained buffer (0.5 M sucrose and 0.125 M phosphate, pH 7.0), and were 50 mM in β-alanine, 1.5 mM in ATP, 2.0 mM in TPP and 1.0 mM in Mg^{2+} added as $MgSO_4$.

TABLE X

The Effect of Added Cofactors and Substrates on Ethylene Production by Crude and Partially Purified Enzyme Powders

Collection period, hours	Treatment	Ethylene, mµl			
		Crude		Partially purified	
		1A	1B	2A	2B
0 - 2	none	N	45	N	90
2 - 20	none	N	89	N	68
20 - 21	none	N	N	N	62
21 - 24	frozen and thawed	N	50	15	57
Total		N	184	15	277
Powder: mgm./ml.		25		1.25	

N = not detectable (less than 5 mµl).

The crude powder was from oat seedlings; the partially purified from wax bean cotyledons.

Reaction mixture: All samples contained buffer (0.5 M sucrose and 0.125 M phosphate, pH 7.0). In addition sample 1B was 1.5 mM in ATP, and sample 2B was 1.5 mM in ATP, 50 mM in β-alanine, 2.0 mM in TPP, 1.0 mM in Mg^{2+} added as $MgSO_4$, 0.5 mM in pyridoxal phosphate, 0.2 mM in Fe^{3+} added as $Fe_2(SO_4)_3$, 50 mM in α-ketoglutarate and 0.17 mM in CoA.

(b) β-Alanine

β-alanine increased ethylene production by approximately 43 percent when added to the crude enzyme powder and ATP (Table XI). Furthermore, production of the volatile by a partially purified enzyme system was greater when the concentration of β-alanine was 50 mM than when it was 10 mM (Table XI). Such evidence indeed suggested that the amino acid was serving as a precursor for ethylene formation by the enzyme powders.

Pertinent to this finding is the fact that the addition of β-alanine stimulated ethylene production by subcellular fractions from tomatoes, particularly in the presence of pyridoxal phosphate, thiamine pyrophosphate and magnesium ion (79).

(c) Cofactors for Transamination

That transamination may be implicated in ethylene production was made apparent by the inhibitory action of cycloserine on ethylene production by a subcellular fraction from tomatoes (79). The involvement of β-alanine in a transamination appeared attractive in view of the apparent capacity of this amino acid as a substrate for ethylene production by enzyme powders.

Addition of pyridoxal phosphate and ferric ion to a crude enzyme system slightly decreased ethylene production (Table XII). Pyridoxal phosphate remains tightly bound to its apoenzyme (108) though, and would probably not be deficient, especially in a crude enzyme preparation. Hence, a boost in enzymatic activity on addition of this cofactor would not necessarily be expected. The slight inhibition might be accounted for by competition between added coenzyme and coenzyme-apoenzyme complex for endogenous β-alanine; no β-alanine was added in

TABLE XI

Effect of β-alanine as a Substrate for Ethylene Production by Enzyme Powders

Collection period, hours	Treatment	Ethylene, mμl			
		Crude		Partially purified	
		1A	1B	2A	2B
0 - 2	none	N	28	27	64
2 - 20	none	62	141		
20 - 21	none	28	23		
21 - 24	frozen and thawed	35	28	77	90
Total		125	220	104	154
Powder: mgm./ml		20		0.75	

N = not detectable (less than 5 mμl).

The crude powder was from wax bean seedlings; the partially purified from wax bean cotyledons.

Reaction mixtures: All samples contained buffer (0.5 M sucrose and 0.125 M phosphate, pH 7.0). Samples 1A and 1B were 1.5 mM in ATP and in addition sample 1B was 50 mM in β-alanine. Samples 2A and 2B were 3 mM in ATP, 2.0 mM in TPP, 1.0 mM in Mg^{2+} added as $MgSO_4$, 0.5 mM in pyridoxal phosphate, 0.2 mM in Fe^{3+} added as $Fe_2(SO_4)_3$, 50 mM in α-ketoglutarate, 70 mM in malonate and 0.17 mM in CoA. In addition sample 2A was 10 mM in β-alanine and 2B 50 mM in β-alanine.

TABLE XII

Effects of Pyridoxal Phosphate and Ferric Ion and of α-ketoglutarate on Ethylene Production by Enzyme Powders

Collection period, hours	Treatment	Ethylene, mμl			
		Crude		Partially purified	
		Control	Pyridoxal Phosphate and Fe^{3+}	Control	α-keto-glutarate
0 - 2	none	56	11	22	22
2 - 20	none	84	90	34	51
20 - 21	none	50	79	28	45
21 - 24	frozen and thawed	62	56	22	48
Total		252	216	106	166
Powder: mgm./ml.		25		5	

The crude powder was from oat seedlings; the partially purified from wax bean cotyledons.

Reaction mixtures: All samples contained buffer (0.5 M sucrose and 0.125 M phosphate, pH 7.0), and were 1.5 mM in ATP. In addition the experimental sample of crude powder was 0.5 mM in pyridoxal phosphate and 0.2 mM in Fe^{3+} added as $Fe_2(SO_4)_3$. Samples of purified powder were in addition 50 mM in β-alanine, 2.0 mM in TPP, 1.0 mM in Mg^{2+} added as $MgSO_4$, 0.5 mM in pyridoxal phosphate, 0.2 mM in Fe^{3+} added as $Fe_2(SO_4)_3$, 0.17 mM in CoA and the experimental sample was 50 mM in α-ketoglutarate as well.

this experiment.

When ∝-ketoglutarate was added to a system of purified enzyme powder and appropriate substrates and cofactors, ethylene production was increased by about 36 percent (Table XII). ∝-ketoglutarate was probably acting as an amino group acceptor for a transamination reaction involving β-alanine.

An enzyme that catalyzes the reversible transamination between β-alanine and ∝-ketoglutarate with the formation of malonic semialdehyde and glutamate respectively has been found present in pig-kidney extracts (109). The reaction has also been shown to be catalyzed by homogenates of mycelial pads from Aspergillus fumigatus (110) and by a fraction separated at 18,000 g from tissue homogenates of brain and liver from mice (111).

The fact that addition of NAD did not stimulate ethylene production by a crude enzyme powder (Table XIII) and by a subcellular particulate fraction from tomatoes (79) is consistent with the involvement of a transaminase rather than a deaminase.

(d) Cofactors for Decarboxylation

Addition of thiamine pyrophosphate and magnesium ion to crude enzyme powder and ATP enhanced ethylene production by about 38 percent (Table XIV), indicating that a decarboxylation was involved in production of the volatile. These cofactors do not remain tightly bound within the system and are probably leached out to some extent during preparation of the crude powder, although cofactors were present endogenously, in concentrations adequate for detectable ethylene production by the crude powder as long as ATP was added. Lipoic acid decreased evolution of the volatile by a crude powder by 73 percent (Table XIV),

TABLE XIII

Effect of Nicotine Adenine Dinucleotide on Ethylene Production by a Crude Enzyme Powder from Oat Seedlings

Collection period, hours	Treatment	Ethylene, mμl	
		Control	NAD
0 - 2	none	56	39
2 - 20	none	34	101
20 - 21	none	79	56
21 - 24	frozen and thawed	107	34
Total		276	230
Powder: mgm./ml.		25	

Samples were from the same batch of enzyme powder.

Reaction mixture: both samples contained buffer (0.5 M sucrose and 0.125 M phosphate, pH 7.0), and were 1.5 mM in ATP, 50 mM in β-alanine, 0.5 mM in pyridoxal phosphate and 0.2 mM in Fe^{3+} added as $Fe_2(SO_4)_3$. The experimental sample was 0.005 mM in NAD in addition.

TABLE XIV

Effects of Thiamine Pyrophosphate and Magnesium Ion and of Lipoic Acid on Ethylene Production by Enzyme Powders

Collection period, hours	Treatment	Ethylene, $m\mu l$			
		Crude		Partially purified	
		Control	TPP & Mg^{2+}	Control	Lipoic Acid
0 - 2	none	56	51	90	45
2 - 20	none	84	163	68	15
20 - 21	none	50	84	62	15
21 - 24	frozen and thawed	62	112	57	N
Total		252	410	277	75
Powder: mgm./ml.		25		0.75	

N = not detectable (less than 5 $m\mu l$).

The crude powder was from oat seedlings; the partially purified from wax bean cotyledons.

Reaction mixtures: All samples contained buffer (0.5 M sucrose and 0.125 M phosphate, pH 7.0). In addition, samples of crude powder were 1.5 mM in ATP and the experimental sample was 2.0 mM in TPP and 1.0 mM in Mg^{2+} added as $MgSO_4$ as well. Samples of purified powder were in addition 3.0 mM in ATP, 50 mM in β-alanine, 2.0 mM in TPP, 1.0 mM in Mg^{2+} added as $MgSO_4$, 0.5 mM in pyridoxal phosphate, 0.2 mM in Fe^{3+} added as $Fe_2(SO_4)_3$, 50 mM in α-ketoglutarate, 0.17 mM in CoA and the experimental sample was 0.02 mM in lipoic acid as well.

suggesting that the decarboxylation is probably nonoxidative.

Additional evidence that a decarboxylation is involved in ethylene production came from experiments with subcellular particulate fractions from tomatoes (79) in which addition of thiamine pyrophosphate and magnesium ion to the particulate fraction and ATP increased evolution of the volatile by 30 percent.

(e) Malonic Acid

The presence of acetate in sufficient concentration to be detected on a sprayed paper chromatogram, even in instances where malonic acid was excluded from the reaction mixture, suggested that malonate was in equilibrium with malonic semialdehyde. Transamination of β-alanine would have yielded malonic semialdehyde which is unstable and was probably transformed to either β-hydroxypropionic acid or malonic acid (Fig. II). Acetate could have been derived from malonate through the action of acetyl CoA carboxylase (112).

Increased ethylene production on addition of malonic acid to a system of crude enzyme powder, β-alanine, thiamine pyrophosphate, magnesium ion and coenzyme A (Table XV) was thought to be attributable to shifts in equilibria favouring production of β-hydroxypropionic acid and ethylene from malonic semialdehyde (Fig. II). Malonate caused an even greater increase in ethylene production when added to partially purified enzyme powder and appropriate substrates and cofactors (Table XV), which is to be expected in view of the fact that endogenous substrates, probably present to some extent in crude enzyme powder, are removed by dialysis during preparation of the purified system. Hence, prior to label studies, it was apparent that malonic semialdehyde was in equilibrium with malonic acid and β-hydroxypropionic acid.

TABLE XV

Effect of Malonic Acid on Ethylene Production by Crude and Partially Purified Enzyme Powders from Wax Bean Cotyledons

Collection period, hours	Treatment	Ethylene, mμl			
		Crude		Purified	
		Control	Malonate	Control	Malonate
0 - 2	none	13	13	N	8
2 - 20	none	19	25	23	23
20 - 21	none	N	11	56	100
21 - 24	frozen and thawed	21	27	96	960
Total		53	76	175	1051
Powder: mgm./ml.		25		0.65	

N = not detectable (less than 1 mμl for analysis of ethylene from the crude system), (less than 5 mμl for analysis of ethylene from the purified system).

The crude and purified systems represent different batches of enzyme powder with differing activities.

Reaction mixture: for the crude system, both samples were 0.125 M in phosphate buffer, pH 7.0, 1.5 mM in ATP, 50 mM in β-alanine, 2.0 mM in TPP, 1.0 mM in Mg^{2+} added as $MgSO_4$ and 0.17 mM in CoA. The experimental sample was also 70 mM in malonic acid. For the purified system, both samples contained buffer (0.5 M sucrose and 0.125 M phosphate, pH 7.0), and were 1.5 mM in ATP, 50 mM in β-alanine, 2.0 mM in TPP, 1.0 mM in Mg^{2+} added as $MgSO_4$, 0.5 mM in pyridoxal phosphate, 0.2 mM in Fe^{3+} added as $Fe_2(SO_4)_3$, 0.17 mM in CoA and 50 mM in α-ketoglutarate. The experimental sample was also 70 mM in malonic acid.

In table XV, it is to be noted that for the crude system the substrate buffer contained phosphate only. In many experiments substrate buffer containing sucrose and phosphate was used; but no difference was noted when sucrose was absent. In some experiments sucrose was omitted to make chromatography of the reaction mixtures easier.

(f) Coenzyme A

Coenzyme A, when added to a system of crude enzyme powder, ATP, thiamine pyrophosphate and magnesium ion boosted ethylene production by approximately 10 percent in one experiment and 20 percent in another (Table XVI). From such evidence, it is not possible to assign a definite role to coenzyme A in this enzyme system, but in view of the proposed pathway for conversion of β-alanine to ethylene (Fig. II) it may be pertinent to such a consideration that β-hydroxypropionyl-coenzyme A and malonic semialdehyde-coenzyme A were reported as intermediates in the oxidation of propionate by Clostridium kluyveri (113).

(g) β-Hydroxypropionic Acid and Acrylic Acid

Propiolactone (a ready source of β-hydroxypropionic acid) at 50 mM in the reaction mixture increased ethylene production (Table XVII, sample A). This increased production is consistent with the conversion of β-alanine (presumably by transamination) through malonic semialdehyde to β-hydroxypropionic acid.

Acrylic acid in the same system including propiolactone, inhibited evolution of the volatile by approximately 70 percent at 75 mM (Table XVII, sample B) and approximately 25 percent at 25 mM. While this behaviour is not expected of an ethylene precursor, the effect of acrylic acid may be through a different mechanism. It is, for example,

TABLE XVI

Effect of Coenzyme A on Ethylene Production by Crude Enzyme Powders from Wax Bean Seedlings

Collection period, hours	Treatment	Ethylene, $m\mu l$			
		A		B	
		Control	CoA	Control	CoA
0 - 2	none	N	28	N	17
2 - 20	none	143	141	23	90
20 - 21	none	35	17	51	28
21 - 24	frozen and thawed	23	34	34	N
Total		201	220	108	135
Powder: mgm./ml.		25		18	

N - not detectable (less than 5 $m\mu l$).

A and B represent different batches of enzyme powder with differing activities.

Reaction mixtures: All samples contained buffer (0.5 M sucrose and 0.125 M phosphate, pH 7.0), and were 1.5 mM in ATP, 2.0 mM in TPP and 1.0 mM in Mg^{2+} added as $MgSO_4$. In addition the experimental samples were 0.17 mM in CoA.

chemically very active, and may attack some other components of the reaction mixture, such as essential sulfide links on enzymes. (Inhibitor experiments with a subcellular fraction from tomatoes have shown that sulfhydryl groups and probably disulfide links are essential for ethylene biosynthesis (78), (79).) It is also interesting in this respect that reactions of ethylene and propylene with atomic sulfur resulting in the formation of cyclic sulfides have been demonstrated (114).

4. A Pathway for Conversion of β-alanine to Ethylene

The evidence discussed above, in conjunction with data obtained from experiments with β-alanine-2-^{14}C, which will be discussed in Chapter VI, suggested a pathway for the conversion of β-alanine to ethylene as illustrated in Fig. II. It was thought likely that β-alanine is transaminated to malonic semialdehyde and thence is converted through β-hydroxypropionic acid and acrylic acid to ethylene. The decarboxylation of acrylate to ethylene could occur by a mechanism analagous to that for the decarboxylation of α-keto acids, as illustrated in Fig. III. Unlike that for the decarboxylation of α-keto acids, this mechanism would not require lipoic acid since there is no oxidation of an aldehyde.

TABLE XVII

Effects of Acrylate and Propiolactone on Ethylene Production by Partially Purified Enzyme Powders from Wax Bean Cotyledons

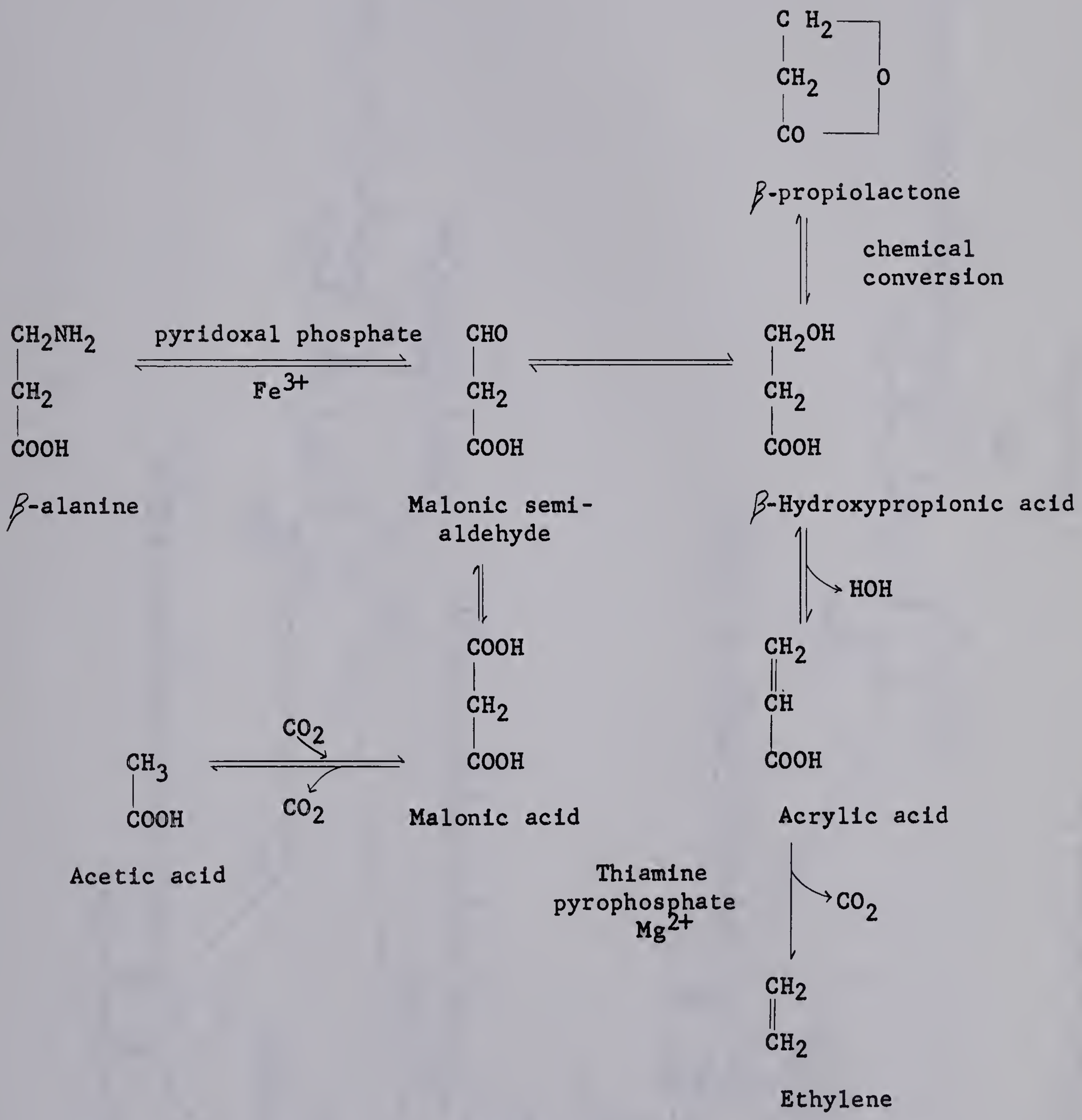
		Ethylene, mμl			
		A		B	
Collection period, hours	Treatment	Control	Propiolactone	Control	Propiolactone and Acrylate
0 - 2	none	22	20	110	16
2 - 20	none	25	20	105	15
20 - 21	none	N	22	50	16
21 - 24	frozen and thawed	23	40	60	15
Total		70	102	325	97
Powder: mgm./ml.		0.5		0.5	

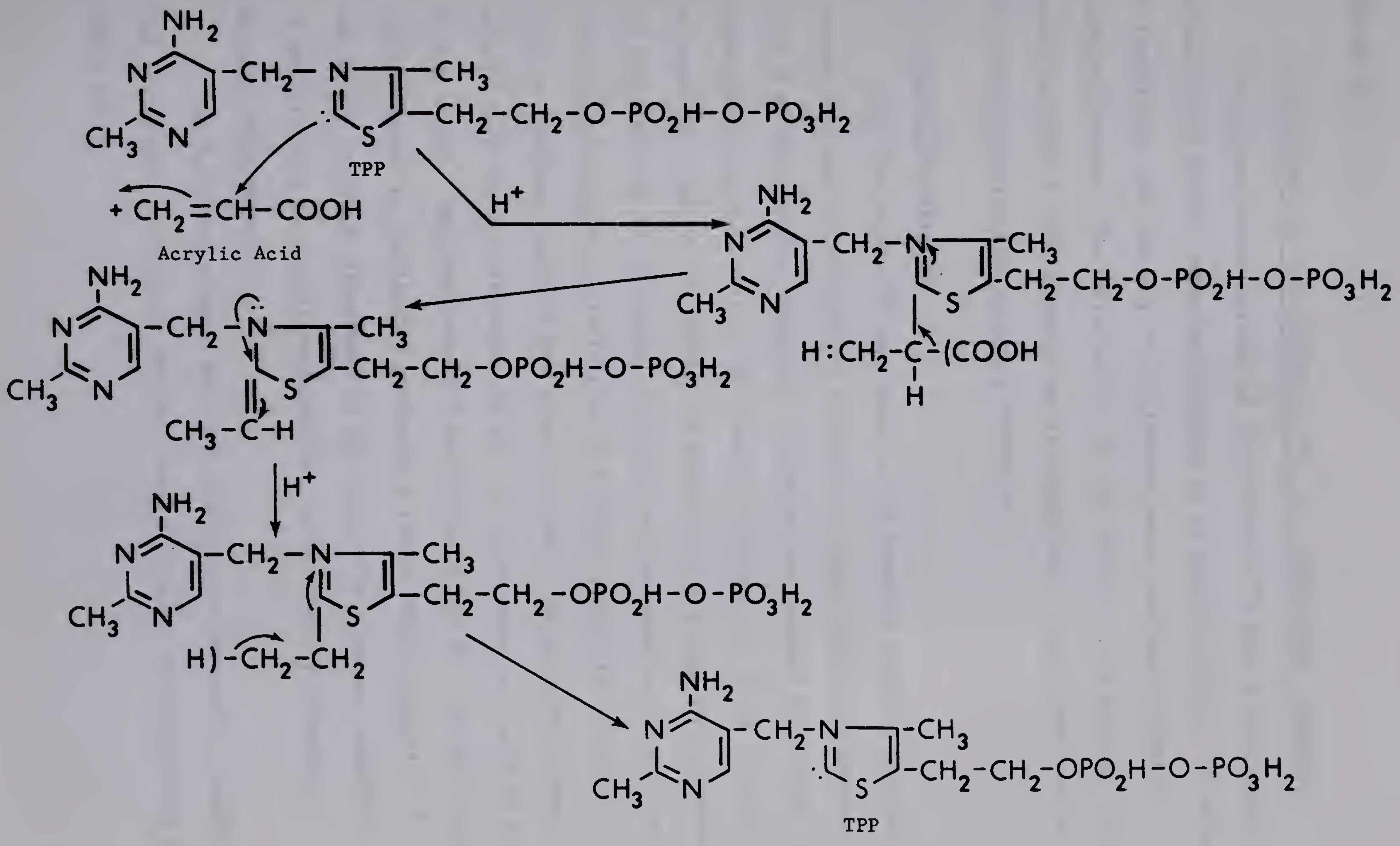
N = not detectable (less than 5 mμl).

A and B denote different batches of enzyme powders with differing activities.

Reaction mixture: All samples were 0.125 M in phosphate buffer, pH 7.0, 3.0 mM in ATP, 50 mM in β-alanine, 2.0 mM in TPP, 1.0 mM in Mg^{2+} added as $MgSO_4$, 0.5 mM in pyridoxal phosphate, 0.2 mM in Fe^{3+} added as $Fe_2(SO_4)_3$, 70 mM in malonic acid, 50 mM in α-ketoglutarate, and 0.17 mM in CoA. In addition, the experimental sample for A was 50 mM in propiolactone and that for B was 75 mM in propiolactone and 75 mM in acrylic acid.

Figure II. Reaction Sequence for Conversion of β-alanine to Ethylene





+ $CH_2=CH_2$

Ethylene

Fig. III. A Possible Reaction Mechanism for the Decarboxylation of Acrylic Acid

Chapter VI

METABOLISM OF β-ALANINE-2-^{14}C TO RADIOACTIVE ETHYLENE

Thin layer chromatography of β-alanine-2-^{14}C with a development solvent of 20 percent ammonium hydroxide in 95 percent ethanol indicated no impurities; the identity of the labeled amino acid was checked by cochromatography. The radioactivity of the isotope in the diluted solution from which aliquots were taken for experiments, was determined by the liquid scintillation counting technique.

1. Radioactive Ethylene

When β-alanine-2^{14}C was added to the complete reaction mixture, labeled ethylene was obtained as indicated in Table XVIII. In samples A and B of Table XVIII, unlabeled β-alanine was reduced from 50 mM, the concentration used in most of the reaction mixtures, to 10 mM in order to lessen dilution of the label. In sample C of the same table however, the concentration of unlabeled β-alanine was increased to 25 mM in an effort to counteract the inhibition of ethylene production by acrylic acid; in addition, malonate was present to shift the equilibria among malonic acid, malonic semialdehyde and β-hydroxypropionic acid in favour of the latter. The lower specific activities of the 21 - 24 hour collections compared to the initial productions (Table XVIII, samples A, B, and C) may implicate adjustment of the equilibria allowing conversion of malonic acid to malonic semialdehyde, rather than β-alanine to malonic semialdehyde.

The following example describes the calculation used to compute the proportions of ethylene derived from β-alanine, as illustrated in Table XVIII.

TABLE XVIII

Radioactivity of Ethylene Metabolized from β-alanine-2-^{14}C
by Partially Purified Enzyme Powders from Wax Bean Cotyledons

Sample	Powder mgm/ml	Collection period, hours	Treatment	Ethylene mμl	DPM	Specific activity DPM/mμl ethylene	Fraction of ethylene derived from β-alanine
A	2.1	0 - 21	none	95.4	2050	13.5	0.41
		21 - 24	frozen & thawed	73.1	225		
B	0.75	0 - 21	none	23	890	12.7	0.38
		21 - 24	frozen & thawed	73	330		
C	1.0	0 - 21	none	28	242	8.4	0.75
		21 - 24	frozen & thawed	22	178		

DPM represent CPM corrected for counting efficiency.

Samples A, B, and C are from different batches of powder with differing activities.

Reaction mixtures: All samples were 0.125 M in phosphate buffer, pH 7.0, 1.5 mM in ATP, 2.0 mM in TPP, 1.0 mM in Mg^{2+} added as $MgSO_4$, 0.5 mM in pyridoxal phosphate, 0.2 mM in Fe^{3+} added as $Fe_2(SO_4)_3$, 70 mM in malonic acid, 50 mM in α-ketoglutarate, 0.17 mM in CoA and contained 67.5μc β-alanine-2-^{14}C. In addition, samples A and B were 10 mM in β-alanine and sample C was 25 mM in β-alanine, 50 mM in propiolactone and 25 mM in acrylic acid.

For Sample A, Table XVIII:

Total label on ethylene produced = 2275 DPM.
Total label on β-alanine added to the reaction mixture = 15,000 x 10^4 DPM.
Therefore, % conversion of label from βalanine to ethylene = $\frac{2275}{15,000 \times 10^4} \times 100 = 0.0015\%$.

Total ethylene produced = 168.5 mμl.
Total β-alanine added to reaction mixture = 0.2 mmoles = potentially 448 X 10^4 mμl of gas.

Therefore, % conversion of β-alanine to ethylene on a molar basis = $\frac{168.5}{448 \times 10^4} \times 100 = 0.0037\%$.

Therefore, comparing these two % conversions, the fraction of ethylene derived from β-alanine = $\frac{0.0015}{0.0037} = 0.41$.

In experiments in which the reaction mixtures for ethylene production contained identical substrate and cofactor complements, but enzyme powders from different batch preparations with differing activities, the specific activities of the ethylene collected were closely similar (Table XVIII, samples A and B). This indicated that the contribution of ethylene from an unlabeled source, presumably malonic acid, was relatively constant in these two experiments. Furthermore, since malonate was the only substrate added besides β-alanine, it may be that the enzyme converting malonic acid to malonic semialdehyde was equally active relative to the transamination of β-alanine, in both enzyme powder preparations. (Bacterial contamination in this ethylene-producing system was found to be negligible throughout a 24 hour period (115).) That the proportion of ethylene derived from β-alanine became greater when the concentration of the amino acid was increased (Table XVIII, sample C), is consistent with the fact that β-alanine at 50 mM relative to 10 mM augmented evolution of the volatile from a partially purified

enzyme system (Table XI).

It is in agreement with the proposed pathway (Fig. II) that addition of 50 mM propiolactone and 25 mM acrylate decreased the specific activity of ethylene labeled by β-alanine-2-^{14}C (Table XVIII, sample C), although the increased concentration of β-alanine probably contributed to the dilution of the label on the volatile as well. An indication of this contribution by β-alanine is gained from the following calculation. For purposes of easier mathematical manipulation in the calculation, although values corresponding to the actual experiments were used for fractions of ethylene derived from β-alanine (Table XVIII), arbitrary values were chosen for the moles of ethylene and β-alanine and for the DPM of β-alanine.

Case 1 - analagous to samples A and B, Table XVIII.

Moles of β-alanine added to the reaction mixture = 850
Total DPM of β-alanine added to the reaction mixture = 5000

Moles of ethylene produced = 500 and 40% (average of samples A and B, Table XVIII) of this was derived from β-alanine.

Therefore DPM of ethylene = $\frac{0.40 \text{ X } 500}{850}$ X 5000 = 1180.

Therefore specific activity of ethylene = $\frac{1180}{500}$ = 2.36 DPM/mole.

Case 2 - analagous to sample C, Table XVIII, where the amount of β-alanine was 2.5 times that in samples A and B.

Moles of β-alanine added to the reaction mixture = 2.5 X 850 = 2120
Total DPM of β-alanine added to the reaction mixture = 5000.

Moles of ethylene produced = 200 and 75% of this was derived from β-alanine.

Therefore DPM of ethylene = $\frac{0.75 \text{ X } 200}{2120}$ X 5000 = 355

Therefore, the specific activity of ethylene = $\frac{355}{200}$ = 1.77 DPM/mole.

Ratio of Case 2/Case 1 = $\frac{1.77}{2.36}$ = 0.75.

Therefore, the specific activity of ethylene was decreased by 25% by the increased concentration of β-alanine.

Application of this information to samples A, B and C, Table XVIII, gives the following results.

> Average specific activity of ethylene for samples A and B = 13.1. Assuming a 25% reduction of the label of ethylene in sample C, as a result of increasing the concentration of β-alanine from 10 mM to 25 mM the specific activity of ethylene would be 0.75 X 13.1 = 9.82.

The experimentally determined specific activity of ethylene for sample C, Table XVIII, was 8.4 which represents a decrease greater than 25 percent, relative to samples A and B. The extra dilution probably reflects ethylene formation from propiolactone and acrylate. In consideration of this calculation, however, it is important to bear in mind that the conversion of β-alanine to ethylene, by a series of steps at least some of which appear to be enzymatic, may not be completely reflected by a proportional computation involving concentrations of only β-alanine and ethylene; a 2.5 fold increase in β-alanine may not be transcripted quantitatively in this system to an effect on the concentration of ethylene.

2. Isolation of the Intermediates by Paper and Thin Layer Chromatography; Quantitative Determinations of their Radioactivities

(a) Chromatography

During the entire procedure of concentration and chromatography, all the acid intermediates were maintained as ethylamine salts since some of the free acids were volatile. In some experiments, ethanol extracts of the reaction mixture were concentrated for chromatography. The fresh ethanol extracts were relatively free of materials interfering with chromatography but after storage of ten days at 5°C a complex probably among ethanol, acrylate and β-hydroxypropionate, judging from its label, was noted to be forming in increasing amounts. No evidence of such a

complex was found in aqueous concentrates.

Any intermediates present as thioesters with coenzyme A were hydrolyzed when the reaction mixtures were basified to pH 10, and heated to 40°C during concentration. Hence in the final concentrate all the acid intermediates were present as their ethylamine salts. Thioesters are more reactive than their analagous oxygen esters (116) and break down readily in alkali with the release of the coenzyme A moiety (117). Moreover, thioesters are much more susceptible to nucleophilic attack by nitrogen bases than by oxygen bases (118); ethylamine was the basifying agent used for the reaction mixtures in this investigation.

Paper chromatography provided adequate separation of the intermediates (Table XIX). Thin layer chromatography furnished higher resolution than did paper chromatography, and more versatility, in that two types of thin layers were used. Typical R_f values for the ethylamine salts of the acids are given in Table XIX, but controls were always run since, with thin layer chromatography, R_f values are somewhat variable.

(b) Radioactivity Determinations

Scanning of the paper chromatograms indicated the presence of labeled malonate and β-hydroxypropionate, but the monitor was not of sufficient sensitivity to detect the radioactivity of other intermediates.

Recovery of administered radioactivity was approximately 50 percent. In addition to losses during the three solution transfers in the procedure, scraping of labeled material from the thin layer chromatography plates to the counting vials was a source of incomplete recovery. Quantitative determinations of the labels were reproducible within a range of approximately 5 percent for the more highly radioactive compounds (β-hydroxypropionate, malonate, and β-alanine) and approximately

TABLE XIX

R_f Values of Ethylamine Salts of Acids in a Pathway for Ethylene Biosynthesis

Development Solvent	Paper chromatography		Thin layer chromatography				
			Cellulose			Silica gel G	
	n-Butanol-H_2O	Ethanol-NH_4OH	n-Butanol H_2O	Isopropanol-NH_4OH - H_2O	Propanol-Methanol NH_4OH - H_2O	Ethanol-NH_4OH	Methanol-NH_4OH
Acrylate	0.35	0.67	0.45	0.57	0.55	0.60	0.84
Acetate	0.27	0.62	0.32	0.51	0.48	0.60	0.84
β-Hydroxy-propionate	0.04	0.41	0.04	0.21	0.34	0.32	0.76
Propiolactone	0.17	0.56	0.22	0.37	0.48	0.48	0.82
Malonate	0.08	0.24	0.09	0.09	0.19	0.09	0.48
α-ketoglutar-ate	0.06	0.29	0.06	0.03	0.28	0.15	0.62
β-alanine	0.02	0.36	0.03	0.21	0.15	0.22	0.49

10 percent for the less radioactive compounds (acrylate, propiolactone, acetate) for the same reaction mixture.

All endogenous substrate molecules were removed initially by dialysis of a solution of the enzyme powder against deionized water for 24 hours prior to lyophilization. Hence with the exceptions of malonic acid, acetic acid and α-ketoglutaric acid, the data in Table XX were interpreted in the same manner as specific activities would be interpreted, and conclusions regarding the sequence of pathway reactions were based on quantitative determinations of the distribution of label. The radioactivities of the intermediates in samples with differing enzyme activities (Table XX) supported the proposed sequence of reactions from β-alanine to ethylene and specific activities of malonate and acetate indicated their formation as a branch pathway (Fig. II). Isolation of the compounds by two-dimensional chromatography and subsequent quantitative determinations of their radioactivities, confirmed the data illustrated in Table XX.

The absence of label on α-ketoglutarate indicated that a conversion of acetate to α-ketoglutarate by enzymes of the tricarboxylic acid cycle did not occur. Further, if acrylic acid had been obtained from acetic acid by a pathway other than indicated (Fig. II), propionic acid would likely have been an intermediate; propionic acid was not detectable in these reaction mixtures. Malonic semialdehyde was not detected, but was probably immediately converted to either malonic acid or β-hydroxypropionic acid.

Burg (61), (64) and Wang, Persyn and Krackov (62), using apple tissue slices and *Penicillium digitatum* respectively, reported that the conversion of glucose carbon atoms to ethylene may follow the route

TABLE XX

Labeling Pattern of Ethylene Formation from β-alanine-2-^{14}C by Partially Purified Enzyme Powders from Wax Bean Cotyledons

	A		B	
Compound(s)	Average DPM per 5 λ aliquot of complete reaction mixture	Average DPM for complete reaction mixture	Average DPM per 5 λ aliquot of complete reaction mixture	Average DPM for complete reaction mixture
Ethylene		2,275		1,220
Acrylate	72	64,800	33	29,700
β-hydroxypropionate	517	465,300	247	222,300
Propiolactone	128	115,000	78	70,200
β-hydroxypropionate +propiolactone	645	580,300	325	292,500
β-alanine	92,757	83,481,300	87,935	79,141,500
α-ketoglutarate	N	N	N	N
	Specific Activities per mmole X 10^{-4}			
Malonate	90		110	
Acetate	11.8		15.4	

DPM = CPM corrected for counting efficiency according to a ^{14}C-quenching curve (for the intermediates) and a ^{14}C standard (for ethylene). A and B denote samples from different batches of enzyme powders with differing activities. N = no count.

Reaction mixture: both samples were 0.125 M in phosphate buffer, pH 7.0, 1.5 mM in ATP, 10 mM in β-alanine, 2.0 mM in TPP, 1.0 mM in Mg^{2+} added as $MgSO_4$, 0.5 mM in pyridoxal phosphate, 0.2 mM in Fe^{3+} added as $Fe_2(SO_4)_3$, 50 mM in α-ketoglutarate, 70 mM in malonic acid, 0.17 mM in coenzyme A and contained 67.5μc β-alanine-2-^{14}C.

involving glycolysis and conversion of acetate to tricarboxylic acid cycle intermediates. Wang, Jacobsen and Tanaka (68) have, as previously indicated in Chapter I, more recently suggested that succinate is transformed through fumarate, malate and acrylate to ethylene. Aspartic acid and glutamic acid increased ethylene production by subcellular particles from tomatoes (79); these amino acids could have been converted to their respective keto acids and hence have become intermediates of the tricarboxylic acid cycle. As well, γ-aminobutyric acid stimulated production of the volatile by the same subcellular system. This acid is known to undergo transamination to succinyl semialdehyde and thence conversion to succinate (119). It is quite plausible that in the experiments performed by the above authors, intermediates of the tricarboxylic acid cycle and their precursors could have been transformed to pyruvate by the action of malic enzyme or phosphoenol pyruvic carboxykinase. Pyruvate could have been converted to ethylene through acetate and malonate in accordance with the pathway proposed in Fig. II. Further in this respect, malonate may have been derived directly from the tricarboxylic acid cycle since there is evidence for the enzymic conversion of oxaloacetate to malonate in plant tissue (112). Gibson and Young (65) recently suggested on the basis of label experiments with Penicillium digitatum that only the 2-C of acetate and the 3-C of pyruvate are incorporated extensively into the ethylene molecule. They also stated (65) that the rate at which ethylene is labeled by various radioactive substrates indicates a pathway from the tricarboxylic acid cycle through pyruvate and acetate to another molecule that then leads to ethylene synthesis. Such evidence supports the proposal that acetate and malonate lead into the pathway for ethylene formation from β-alanine. Burg and

Burg (63) reported that in apple tissue slices a 200 minute collection of ethylene labeled by acetate-2-^{14}C had a higher specific activity than that labeled by acetate-1-^{14}C; this further supports the evidence for formation of the symmetrical molecule, malonic acid, via acetyl CoA carboxylase in the pathway from acetate to ethylene. Ethanol has been shown to promote increased production of ethylene by subcellular particles from tomatoes (79). Pertinent to this may be the fact that ethanol could have been metabolized to acetyl-coenzyme A (120) and thus have entered the pathway for ethylene production.

Recent label studies with Penicillium digitatum (16) indicated a very high efficiency of transformation of C-3 of serine into the ethylene molecule. It is significant in this respect that serine can be converted by transamination to pyruvate and subsequently C-3 of serine becomes C-2 of acetate. Subcellular fractions from tomatoes also produced more ethylene in the presence of added serine (79).

Jacobson and Wang (70) detected labeled ethylene when Penicillium digitatum was incubated in medium containing acrylate-2(3)-^{14}C.

Lieberman, Mapson, Wardale and Kunishi (121) have reported labeling of ethylene by methionine-3(4)-^{14}C in apple tissue slices. Methionine can be converted to propionic acid by known metabolic routes; the 3-C and 4-C of methionine became C-2 and C-3 respectively of propionic acid. It is known that propionyl-CoA can be transcarboxylated with oxaloacetate to form methylmalonyl-CoA, which can then be converted by methylmaloynl-CoA isomerase to succinyl-CoA, and presumably through acetate to ethylene. In Clostridium propionicum, there is indication for the conversion of propionyl-CoA to acrylyl-CoA (122). (Both Wang (70) and Burg (63) have obtained radioactive ethylene from labeled pro-

pionic acid in Penicillium digitatum and apple slices respectively. As well, propionate was found to enhance ethylene production by subcellular particulate fractions from tomatoes (79).) Hence, metabolism of methionine-3(4)-^{14}C could have occurred through propionate to ethylene.

It has been reported that mitochondria from peanut cotyledons supplemented with ATP and coenzyme A oxidized propionate through β-hydroxypropionate to acetate and carbon diozide (123), (124). The carboxyl carbon of propionate was converted to carbon dioxide and carbons 2 and 3 became the methyl and carboxyl carbons respectively of acetate. Malonic semialdehyde and malonyl coenzyme A were proposed as intermediates in the breakdown of β-hydroxypropionate.

Partial purification of enzymes catalyzing the reversible conversions of acrylyl-coenzyme A to β-hydroxypropionyl-coenzyme A and of β-hydroxypropionate to malonic semialdehyde in pig kidney tissue have been reported (125). As well, pig heart and liver extracts and also Propionibacterium shermanii contain the latter enzyme. A requirement of NAD for the β-hydroxypropionate dehydrogenase reaction was not fulfilled by NADP and the enzyme was inactive with β-hydroxypropionyl-coenzyme A. Vagelos and Earl (113) partially purified an enzyme catalyzing an NADP nucleotide-specific dehydrogenation of β-hydroxypropionyl-coenzyme A; the enzyme was inactive with free β-hydroxypropionate. It is interesting in this respect that ethylene production by enzyme powders from wax bean cotyledons was augmented in the presence of reduced NADP (115). Moreover, the author found increased ethylene production by the enzyme powders when coenzyme A was added to the reaction mixture.

Evidence has been presented that indicates a conversion of malonate to malonic semialdehyde by enzyme powders active in ethylene produc-

tion (Table XV). Since such a reduction, like that catalyzed by phosphoglyceraldehyde dehydrogenase and the coenzyme A-dependent aldehyde dehydrogenase from Clostridium kluyveri and Escherichia coli (126), would require energy, it may be pertinent that ethylene production by enzyme powders was increased in the presence of ATP. Vagelos (126) prepared from Clostridium kluyveri an enzyme system that contained β-hydroxypropionyl-coenzyme A dehydrogenase and an NADP-sensitive malonyl semialdehyde-coenzyme A dehydrogenase; separation of the two enzymes was not achieved. Although he was unable to detect reduction of malonyl-coenzyme A to malonyl semialdehyde-coenzyme A by this enzyme preparation, the sensitivity of the methods used for assessing the formation of the latter compound was not capable of picking up a reversal of less than 5 percent; the concentrations of reactants were not stated for these experiments. Attempts to ascertain the extent of reaction included following the concentration of reduced NADP at 340 mμ and chromatography of the hydroxamate derivative of β-hydroxypropionyl-coenzyme A, the presence of which would reflect formation of malonyl semialdehyde-coenzyme A. In view of the low levels of ethylene produced by the enzyme powders, a small conversion of malonic acid to malonic semialdehyde would probably have a measurable effect on ethylene production. As well, it is to be kept in mind that there are often inherent differences in metabolic pathways between bacteria and plants. It is also interesting to speculate that a more efficient conversion of malonic acid to malonic semialdehyde is characteristic of metabolism during senescence and hence related to the high ethylene production associated with aging.

Catalysis of the carboxylation of acetyl-coenzyme A to malonyl-coenzyme A by Saccharomyces cerevisiae (127) and pig heart extracts (128)

has been reported. As well, evidence has indicated the breakdown of malonyl-coenzyme A to carbon dioxide and acetyl-coenzyme A by a particulate fraction from bush bean leaves (129) and peanut mitochondria (130). Such information supports the positions of malonate and acetate as a side branch in the proposed pathway for conversion of β-alanine to ethylene (Fig. II).

Chapter VII

VARIATION OF MALONIC ACID LEVELS DURING THE LIFE CYCLE OF WAX BEAN COTYLEDONS

When malonic acid was added to the reaction mixtures of crude or partially purified enzyme powders prepared from wax bean cotyledons, ethylene production was augmented (Table XV). It was suspected that the increased concentration of malonate boosted ethylene production by allowing the formation of β-hydroxypropionic acid from malonic semialdehyde to be favoured (Fig. II). Furthermore, it was thought possible that endogenous levels of malonate might indeed be a part of a control mechanism for ethylene biogenesis.

Experiments to determine the endogenous levels of malonic acid in wax bean cotyledons throughout germination were conducted. For these determinations, 5 λ aliquots of the concentrated extracts from cotyledon tissue were developed in a solvent of chloroform, ethanol (95 percent) and formic acid (90 percent) in the volumetric ratio of 50:50:1 by thin layer chromatography. Development in this solvent system provided adequate isolation of malonic acid (Table XXI). A further indication of the R_f value, in this solvent system, of malonic acid relative to those of other acids that might have been present in the extract, is given in a publication by Stark, Goodman and Owens (100). In addition, a mixture of nucleotides did not move in the solvent system. Interference from sugars was minimized by extraction of dried cotyledon tissue with 95 percent ethanol (Chapter II). (The solubility of malonic acid in 95 percent ethanol is 57 gm. per 100 ml. at 20°C). The results of thin layer chromatography were confirmed by two dimensional chromatography. Chromatograms that had been previously developed in the "chloro-

TABLE XXI

R_f Values of Acids and Amino Acids on Cellulose Thin Layer Chromatograms

Acid	R_f Value	Amino Acid	R_f Value
Fumaric	0.92	Isoleucine	0.5
Pyruvic	0.92	Leucine	0.5
Succinic	0.92	Valine	0.35
Propiolactone	0.92	Cysteine	0.25
Malonic	0.83	Phenylalanine	0.13
Malic	0.65	Tryptophan	0.13
β-hydroxy-propionic	0.60	Histidine	0.1
Oxaloacetic	0.52	Methionine	0.1
		Alanine	0.09
		β-alanine	0.08
		Threonine	0.08
		Serine	0.03

Development solvent: chloroform, ethanol (95 percent) and formic acid (90 percent) in the volumetric ratio of 50:50:1.

form-ethanol solvent" were run in a second direction with a solvent of water-saturated n-butanol; in the latter solvent, malonic acid had an R_f value of 0.62.

It is to be noted that determinations of the amounts of malonic acid in 5 λ aliquots from the same extract, varied as much as 15 percent of the average level (0.054 X 10^{-2} mmoles) in 5 λ aliquots of extracts from cotyledon tissue harvested 9 days after planting; the level of acid in the "9 day tissue" was the largest average amount detected. Variations among extracts from different batches of tissue of the same age were as low as 10 percent and as high as 33 percent of the same average value (0.054 X 10^{-2} mmoles). The high variation obtained in determinations of low levels of malonic acid reflected the lengthy and complex extraction procedure that was necessary (Chapter II). Also, it is difficult to exactly duplicate the physiological stages of development of tissues at the time of harvesting, even under nearly identical growing conditions. In spite of these variations, however, a plot of average values indicates a definite trend towards increasing levels of malonate in the cotyledons as they aged (Fig. IV).

The author is aware of the fact that the experimental variations would have been less significant if malonic acid had been isolated, by paper chromatography, from larger aliquots than could be handled by thin layer chromatography, but was unable to attempt these experiments prior to submission of the thesis.

Determinations of the levels of malonic acid in cotyledons indicated progressively increasing amounts throughout germination; the increment paralleled to some extent the increased production of ethylene in senescing cotyledons (Fig. IV). Ethylene production by tissue harvested later than 8

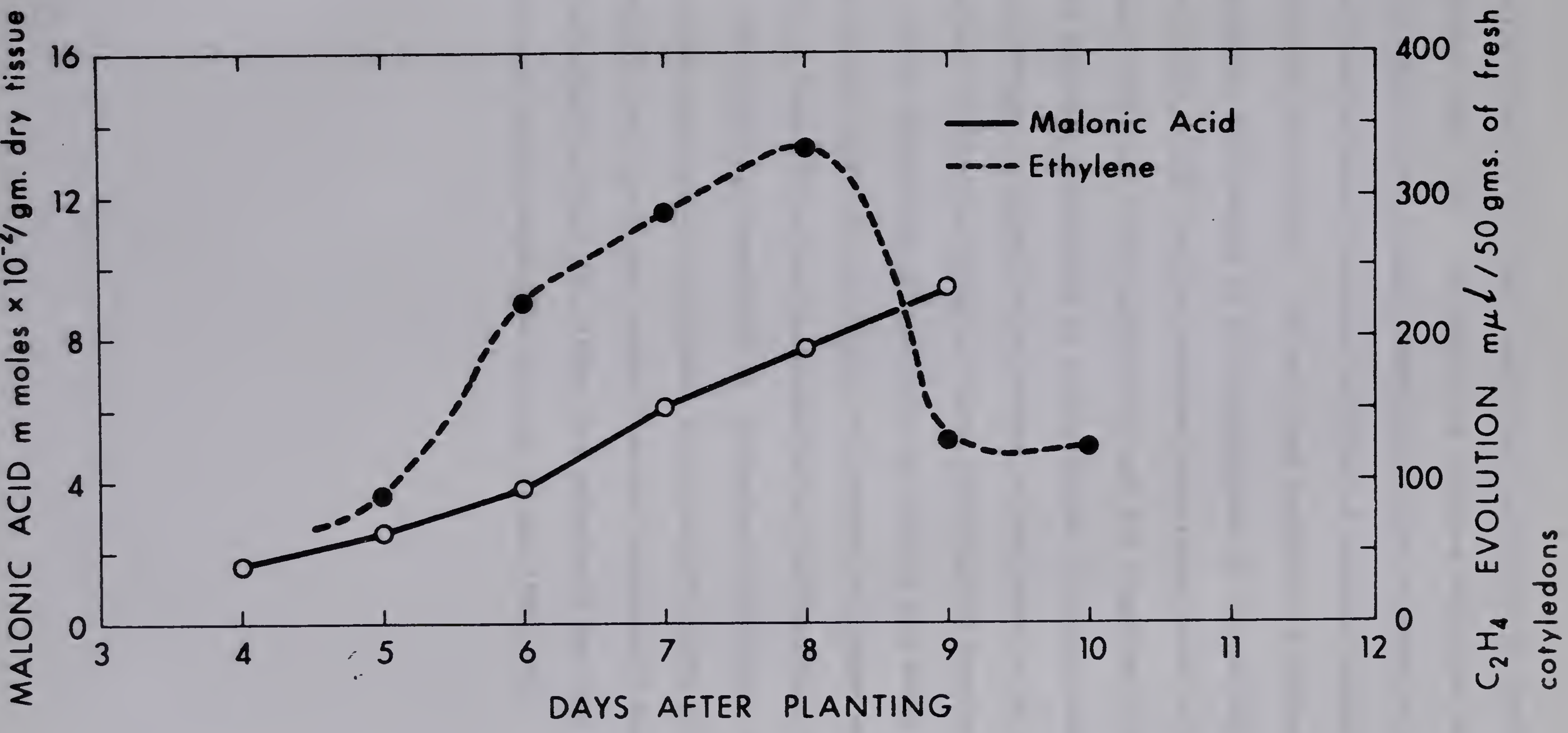


Fig. IV. Ethylene Production by Wax Bean Cotyledons in relation to Endogenous Malonic Acid Levels

days after planting decreased even though malonic acid levels continued to increase (Fig. IV). The decline in ethylene evolution corresponded with the stage of development immediately prior to death of the tissue and may have reflected decreased activity of enzymes responsible for biosynthesis of the volatile. As well, the synthesis of various factors (for example, ATP) needed in the biosynthesis of the gas may have decreased. Duperon (131), (132) has also detected increasing levels of malonate in wax bean cotyledons during germination and has suggested (133) that the acid is probably synthesized by carboxylation of acetyl-coenzyme A. The percentages, obtained by the author, of malonate in dry cotyledon tissue, are in the same range as those determined by Duperon.

It is interesting to speculate, in view of the above evidence, that with the onset of senescence, the presence of larger malonate pools inhibits a drain of malonic semialdehyde for the formation of malonic acid, thus promoting conversion of β-alanine to ethylene. Moreover, in view of the association of ethylene evolution with aging of tissue, it is possible that β-alanine arises from the breakdown of nucleic acids, (see Introduction, page 15), since this amino acid has been shown to be a product of uracil catabolism in plant tissue (90), (91).

Chapter VIII

A NON-ENZYMATIC MODEL FOR ETHYLENE PRODUCTION FROM β-ALANINE

Ethylene was produced by a heat-catalyzed Snell reaction (101) among β-alanine, pyridoxal hydrochloride and cupric ion. Such a system serves as a model for an enzymatic production of the volatile, supporting β-alanine as a precursor in the biosynthesis of ethylene.

Evolution of ethylene occurred when pyridoxal hydrochloride β-alanine and cupric ion were autoclaved, but in the absence of any one component or in the absence of heat, no ethylene was produced (Table XXII). Conversion to ethylene was 0.0014 percent of the theoretical. Increases in the absolute amounts of the reaction mixture components elevated ethylene production (Table XXII, sample 14). Treatment of the gaseous phase with mercuric perchlorate solution, which allowed the ethylene to complex with mercury (94), eliminated contamination from other volatiles produced by heat-induced decomposition of the rubber stoppers; ethylene was subsequently reliberated from the complex with mercury by the addition of lithium chloride. The progress of the Snell reaction was confirmed by determinations of the concentrations of pyridoxal hydrochloride both before and after autoclaving. Use of an all-glass reaction vessel and analysis of the complete gaseous mixture revealed the presence of ethylene only when all three reaction components were autoclaved together.

TABLE XXII

Ethylene Production by a Snell Reaction among β-alanine, Pyridoxal hydrochloride and Cupric Ion

Sample number	Standard solutions, mls. Pyridoxal hydrochloride	β-alanine	Cu^{2+}	Autoclaved	Ethylene $m\mu l$	Pyridoxal hydrochloride, μ moles
1	2	2	1	+	20	59.7
2	2	2	1	-	N	65.0
3	2	2	-	+	N	63.2
4	2	2	-	-	N	65.0
5	2	-	-	+	N	65.0
6	2	-	-	-	N	65.0
7	-	2	-	+	N	-
8	-	2	-	-	N	-
9	2	-	1	+	N	64.7
10	2	-	1	-	N	63.2
11	-	2	1	+	N	-
12	-	2	1	-	N	-
13	-	-	-	+	N	-
14	20	20	10	+	395	

N = not detectable (less than 5 $m\mu l$).

Samples 1 - 13 were made up to 5 ml. volumes with 0.2 M acetate buffer, pH 4.5, where necessary. Autoclaving was for 30 minutes at 20 lbs. steam pressure, with fast exhaust.

Standard solutions: pyridoxal hydrochloride was 0.0325 M in 0.2 M acetate buffer at pH 4.5; β-alanine was 1.0 M and Cu^{2+} was 0.625 mM added as $CuSO_4$, in the same buffer.

Chapter IX

GENERAL CONCLUSIONS

Considerable interest has arisen concerning the formulation of a metabolic pathway for production of ethylene. For some time, there has been a need in this field of study for a biochemically simplified system for production of the volatile. The present investigation has comprised the preparation of an enzyme powder capable of catalyzing ethylene production, and further exploration by substrate and cofactor studies with this enzyme system of a pathway for synthesis of the volatile; this is the first report of a moderately refined enzyme powder for ethylene production. In addition, radioactive tracer studies were conducted and further support was gained for a proposed sequence of reactions for conversion of β-alanine to ethylene.

Crude enzyme powders, active in ethylene production, were prepared from subcellular particulate fractions of oat seedlings, *Avena sativa*, wax bean seedlings, *Phaseolus vulgaris*, and wax bean cotyledons. A suspension of the particulate fraction was frozen and thawed to promote particle-disruption. Lyophilization of an ammonium sulphate precipitation from this disrupted suspension, yielded a crude enzyme powder. Removal of lipids and subsequent ammonium sulphate fractionation effected a partially purified enzyme powder, active in ethylene production.

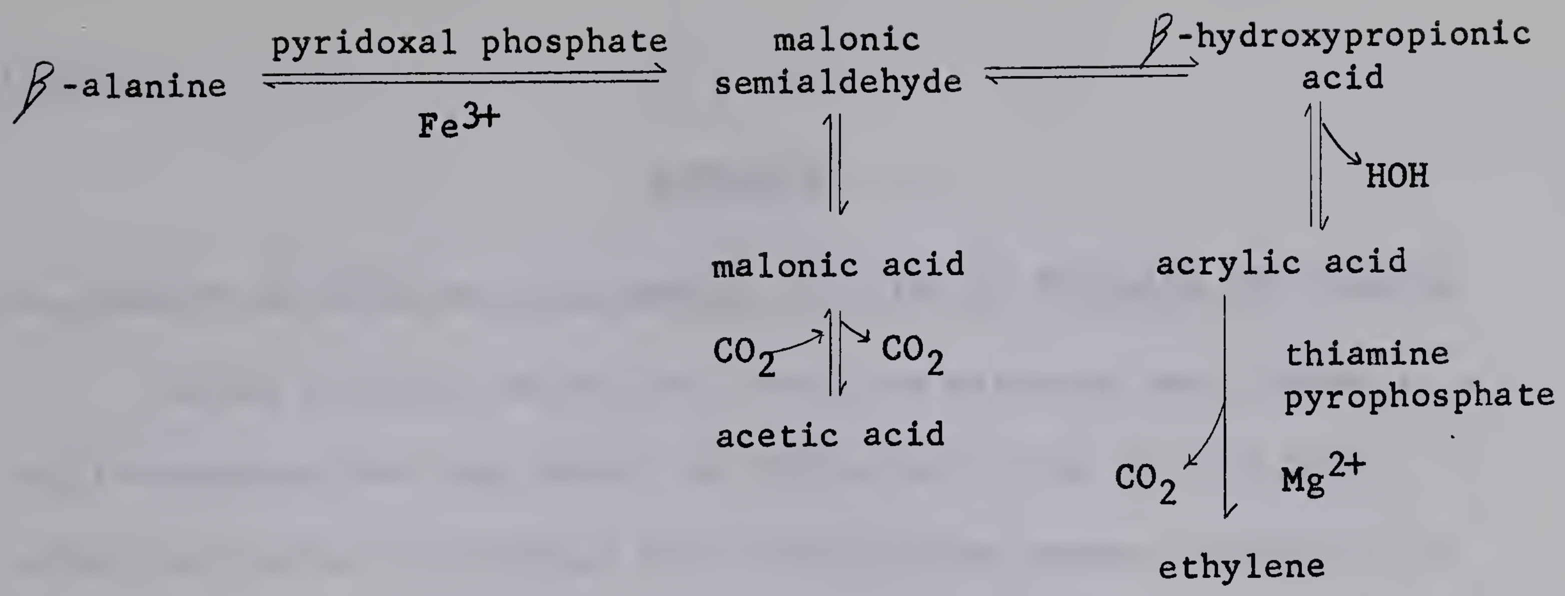
An added source of energy was found to be requisite for catalysis of ethylene formation by these powders; ATP sufficed in this respect. As well, β-alanine proved to be a precursor of the volatile. The elevation of ethylene production on addition of α-ketoglutarate was consistent with a role for transamination in the conversion of β-alanine to ethylene; the keto acid probably served as an amino group acceptor.

Thiamine pyrophosphate and magnesium ion promoted ethylene production from β-alanine, thus implicating the involvement of a decarboxylation. In view of the inhibitory effect of lipoic acid, it was thought probable that the decarboxylation was non-oxidative. Coenzyme A also enhanced ethylene production.

Increased ethylene production on addition of propiolactone, an immediate source of β-hydroxypropionic acid, was consistent with transamination of β-alanine to malonic semialdehyde and subsequent transformation through β-hydroxypropionic acid and acrylic acid to ethylene. An unexpected inhibition of ethylene evolution by acrylic acid may have been attributable to interference, by added acrylate, with essential sulfide groups on enzymes.

Ethylene production by the enzyme powders was enhanced when malonic acid was added to a system of enzyme powder in buffer, ATP, β-alanine, coenzyme A and the cofactors for transamination and decarboxylation. It became apparent that the formation of malonate and acetate constituted a branch pathway in the conversion of β-alanine to ethylene, and that added malonate probably prevented, by product inhibition, a drain in this direction from the main pathway. In view of the peak in ethylene production by wax bean cotyledons during their senescence, the finding of augmented levels of malonic acid in the cotyledons during this stage of development, supported a postulation that such a mechanism of product inhibition by malonate may be operative in the in vivo production of ethylene from β-alanine.

Experimental evidence allowed the proposal of the following pathway for conversion of β-alanine to ethylene.



When β-alanine-2-^{14}C was added to the enzyme systems, labeled ethylene was obtained. Quantitative determinations of the radioactivities of the intermediates supported the proposed sequence of reactions for the transformation of β-alanine to ethylene. Specific activities of malonate and acetate were consistent with their proposed involvement as a branch pathway.

Ethylene was produced by a heat-catalyzed Snell reaction among β-alanine, pyridoxal hydrochloride and cupric ion. Such a system serves as a model for enzymatic production of the volatile and further supports the role of β-alanine as a substrate for ethylene production.

Considerable experimentation regarding a pathway for ethylene synthesis has been conducted in other laboratories. Many of the reported results are consistent with the operation of a pathway of transformation of β-alanine to ethylene as proposed in this thesis. Such data may as well, however, indicate independent systems for formation of the volatile.

Chapter X

APPENDIX

1. Release of Ethylene from Aqueous Solution by Freezing and Thawing

During ethylene collections, reaction mixtures were frozen in a dry ice-acetone bath and thawed at 40°C prior to the 21 - 24 hour collection period, to release the volatile from aqueous solution. In order to assess the effectiveness of this treatment, experiments were carried out in which a known quantity of ethylene was injected through a rubber septum, and under the surface of 40 ml. of 0.125 M phosphate buffer, pH 7.0, in a closed container; there was a limited gas volume (approximately 2 ml.) over the solution. The mixture was stirred for 1 hour at room temperature. (Ethylene collections from suspensions, in buffer of the same composition, of enzyme powders and their required substrates and cofactors, were routinely conducted at room temperature.) Subsequently, all ethylene that had not gone into solution was removed from the air space over the solution. Two 20 ml. portions of the buffer containing ethylene in solution were pipetted into different reaction flasks and two ethylene collections, each of one hour duration, were taken from one of these without treatment, and from the other with freezing and thawing prior to collection. For freezing and thawing, the stoppered flask was frozen in a dry ice-acetone bath and thawed by agitation in a 40°C water bath, as was done routinely prior to the 21 - 24 hour ethylene collections from enzyme powders.

Greater amounts of ethylene were obtained from the treated samples than from the untreated (Table XXIII). In these experiments, both samples within a trial were of equal volume and contained equal amounts

TABLE XXIII

Release of Ethylene from Aqueous Solution
by Freezing and Thawing

Trial No.	Collection period, hours	Ethylene, mμl Untreated Sample	Ethylene, mμl Treated Sample
A	0 - 1	9	29
	1 - 2	62	92
	Total	71	121
B	0 - 1	0	30
	1 - 2	25	28
	Total	25	58

Both samples within a trial contained equal amounts of ethylene in 20 ml. of 0.125 M phosphate buffer, pH 7.0 but the amounts differed between trials A and B. Treatment constituted freezing in a dry-ice-acetone bath and thawing at 40°C, and was given to the treated sample prior to each ethylene collection period.

of ethylene in solution, but the amounts of the volatile in solution varied between trials. A lower ethylene evolution in the first hour of collection relative to the second (Table XXIII), may reflect the time required for equilibration of the ethylene between the solution and the air space above, and its uniform distribution within the air space itself, for collection was by a continuous flow of purified air, with entry and exit at the top of the enclosed air space. A complex, that forms below 18.7°C (134), between ethylene and water would decompose during the thawing treatment.

2. Establishment of the Identity of Ethylene

The identity of ethylene was established by gas chromatographic analysis with the unit constructed by Chandra (93). Two types of columns, activated alumina coated with silicone and squalane-coated firebrick, were used. In all cases with both types of columns, the retention times for collected samples and known standard samples of ethylene were exactly identical, and distinct from those of compounds with closely related structures. In addition, when a Perkin-Elmer 811 gas chromatograph, fitted with an activated alumina column (37.2 cm. x 0.5 cm.), was used with the flow rates and temperature settings of Stinson and Spencer (115), retention times of 34.3 seconds were consistently identical for collected samples and standard samples of ethylene. The validity of such identification is heightened in view of the fact that Burg and Burg (81) were able to distinguish ethylene-like substances from ethylene by gas chromatography with an activated alumina column. Confirmation of the identity of ethylene was established by mass spectrometric analyses. A spectrum of the volatile evolved from an enzyme powder is illustrated in Fig. V and of the volatiles produced by a heat-

catalyzed Snell reaction among β-alanine, pyridoxal hydrochloride and cupric ion, in Fig. VI. The presence at masses 26 and 27 of peaks of magnitudes in the ratio 0:96:1 (Figures V and VI) is representative of ethylene.

3. Nitrogen values

Nitrogen determinations for the enzyme powders were carried out by the "boric acid method" as outlined by Ogg (135). Representative nitrogen values were 0.15 mgm. nitrogen per mgm. powder for the crude system and 0.11 mgm. nitrogen per mgm. powder for the partially purified system. Since the crude powders were obtained by an ammonium sulphate precipitation (Chapter II), nitrogen values for the crude powders are not entirely representative of endogenous nitrogen because of varying degrees of contamination by ammonium sulphate.

4. Standard curves and a Quenching Curve for Carbon-14

For determinations of specific activities of the ethylamine salts of malonate and acetate, their concentrations were ascertained spectrophotometrically. The salts were complexed with benzidine hydrochloride and copper, and the absorbancy of this complex was read at 242 mμ on a Beckman D.U. spectrophotometer (98). Standard curves are illustrated for the ethylamine salt of acetate (Fig. VII) and the ethylamine salt of malonate (Fig. VIII). Experimental readings for which these curves were used, were within the absorbancy range of 0.13 - 0.2. For determinations of the concentrations of malonic acid, a standard curve (Fig. IX) was prepared as above, only the acid rather than its salt was used. Experimental readings for which this curve was used, were within the absorbancy range of 0.005 to 0.058.

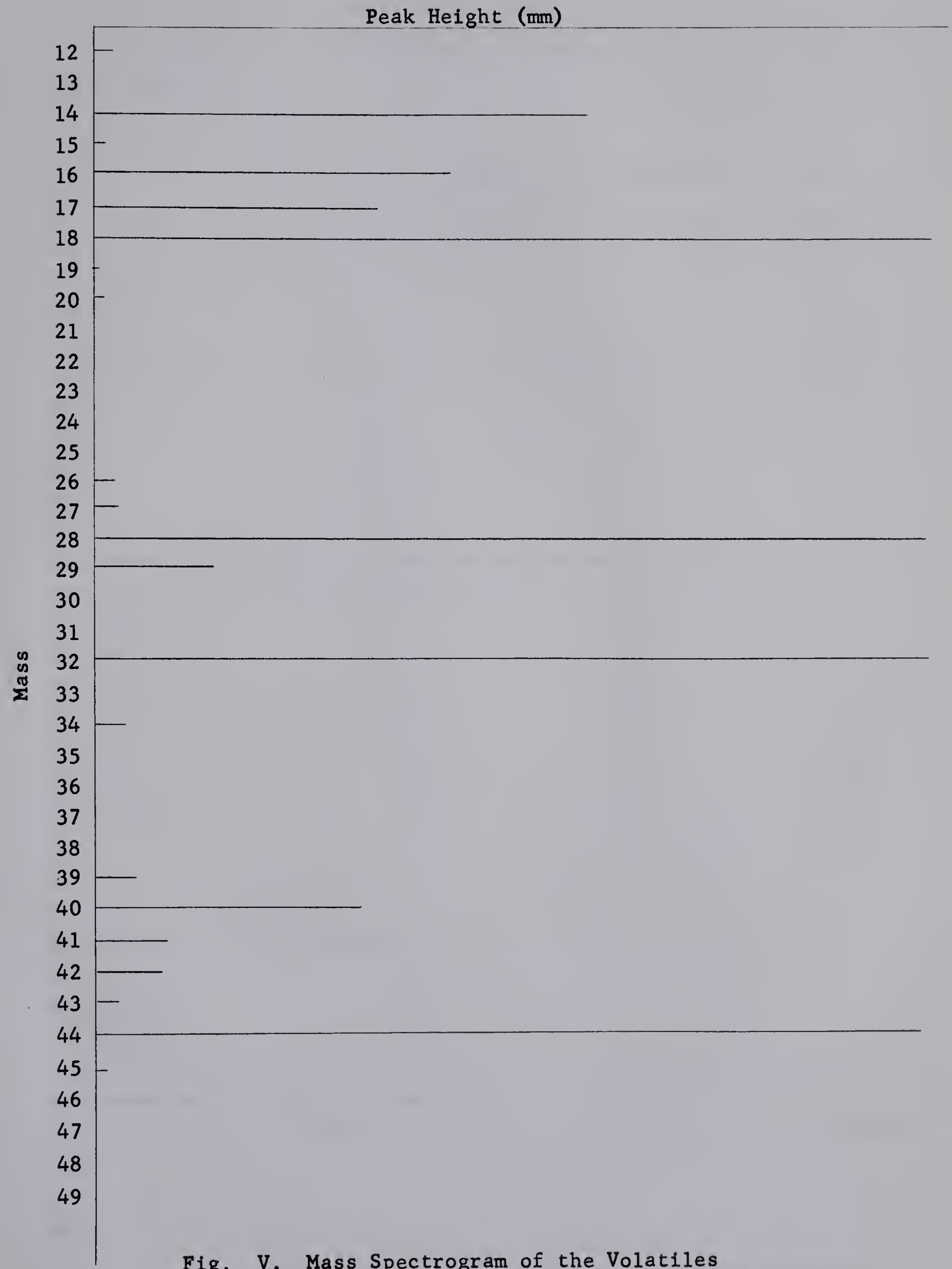


Fig. V. Mass Spectrogram of the Volatiles Produced by an Enzyme Powder

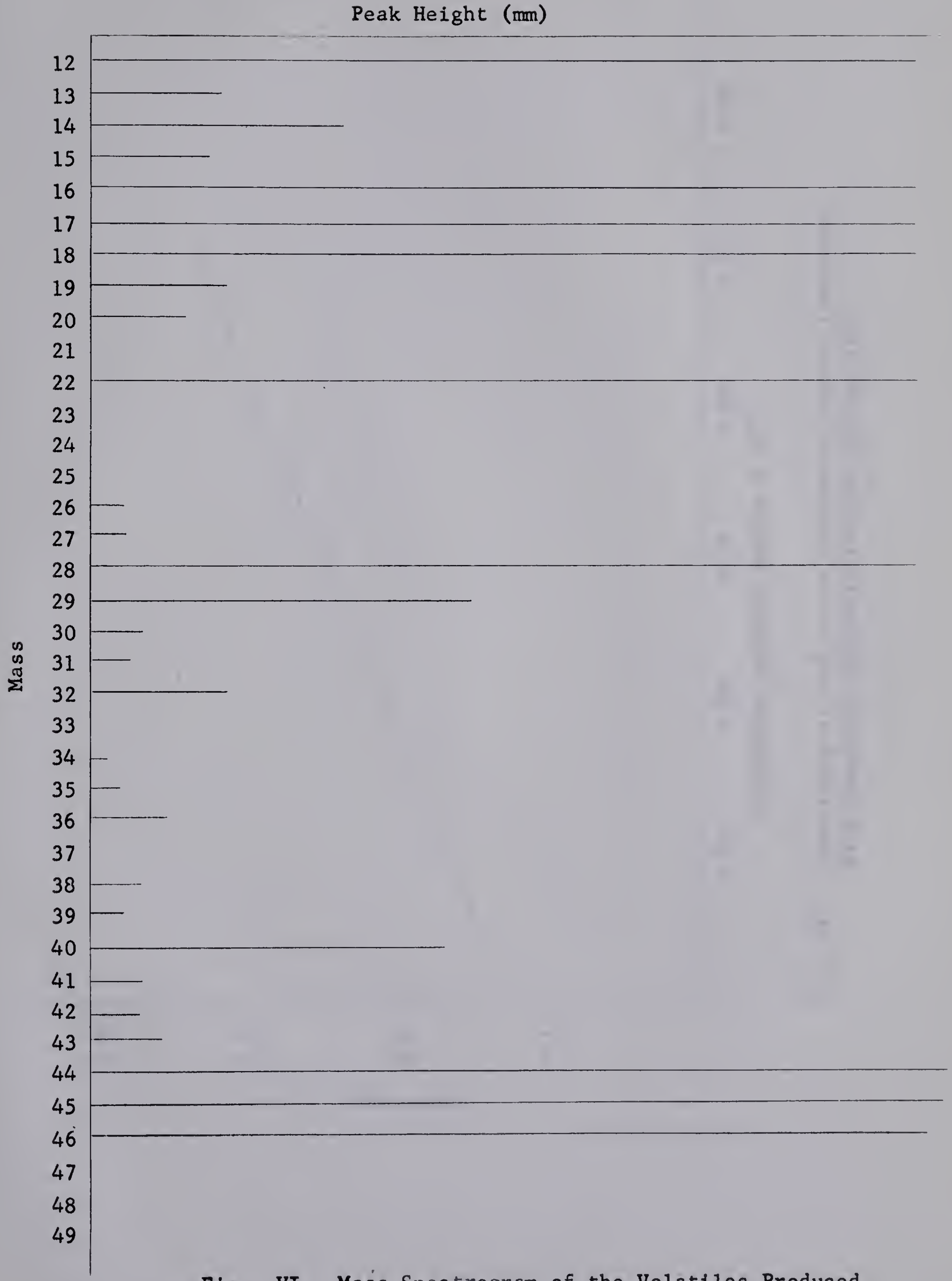


Fig. VI. Mass Spectrogram of the Volatiles Produced by a Snell Reaction among β-alanine, Pyridoxal hydrochloride and Cupric ion.

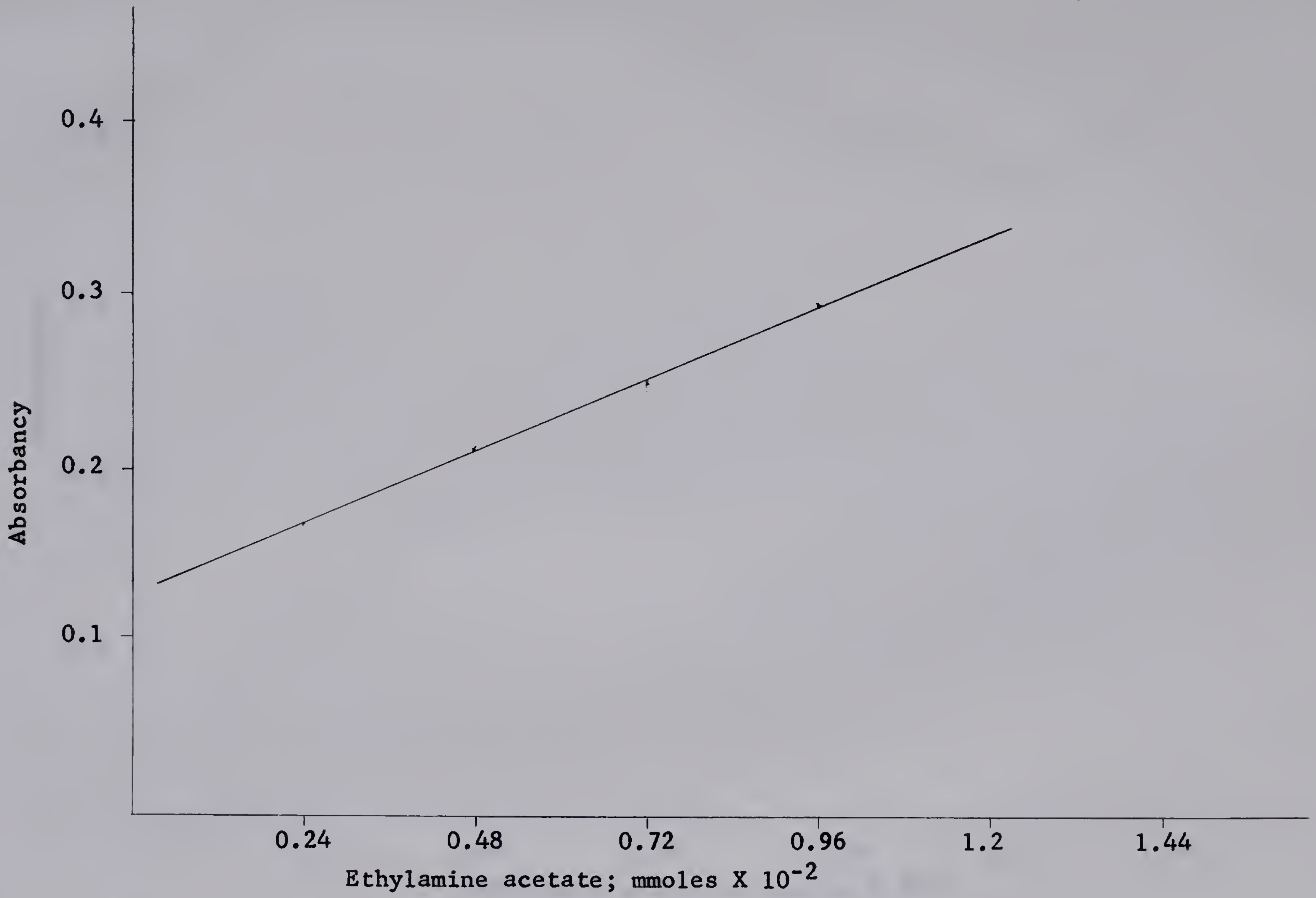


Fig. VII. Absorbancy at 242 mμ of Ethylamine Acetate Complexed with Benzidine Hydrochloride and Cuprous Ion

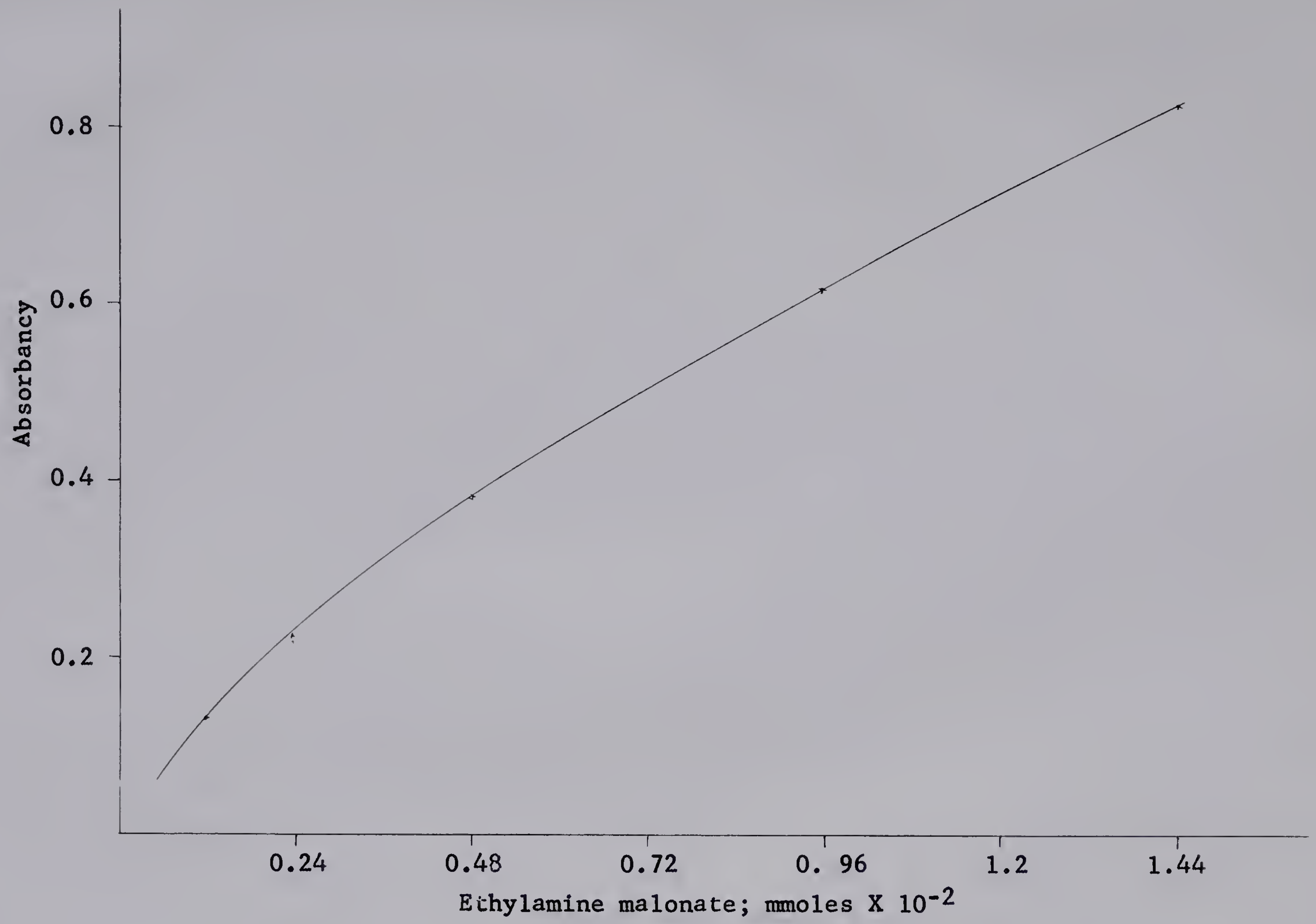


Fig. VIII. Absorbancy at 242 mμ of Ethylamine Malonate Complexed with Benzidine Hydrochloride and Cuprous Ion

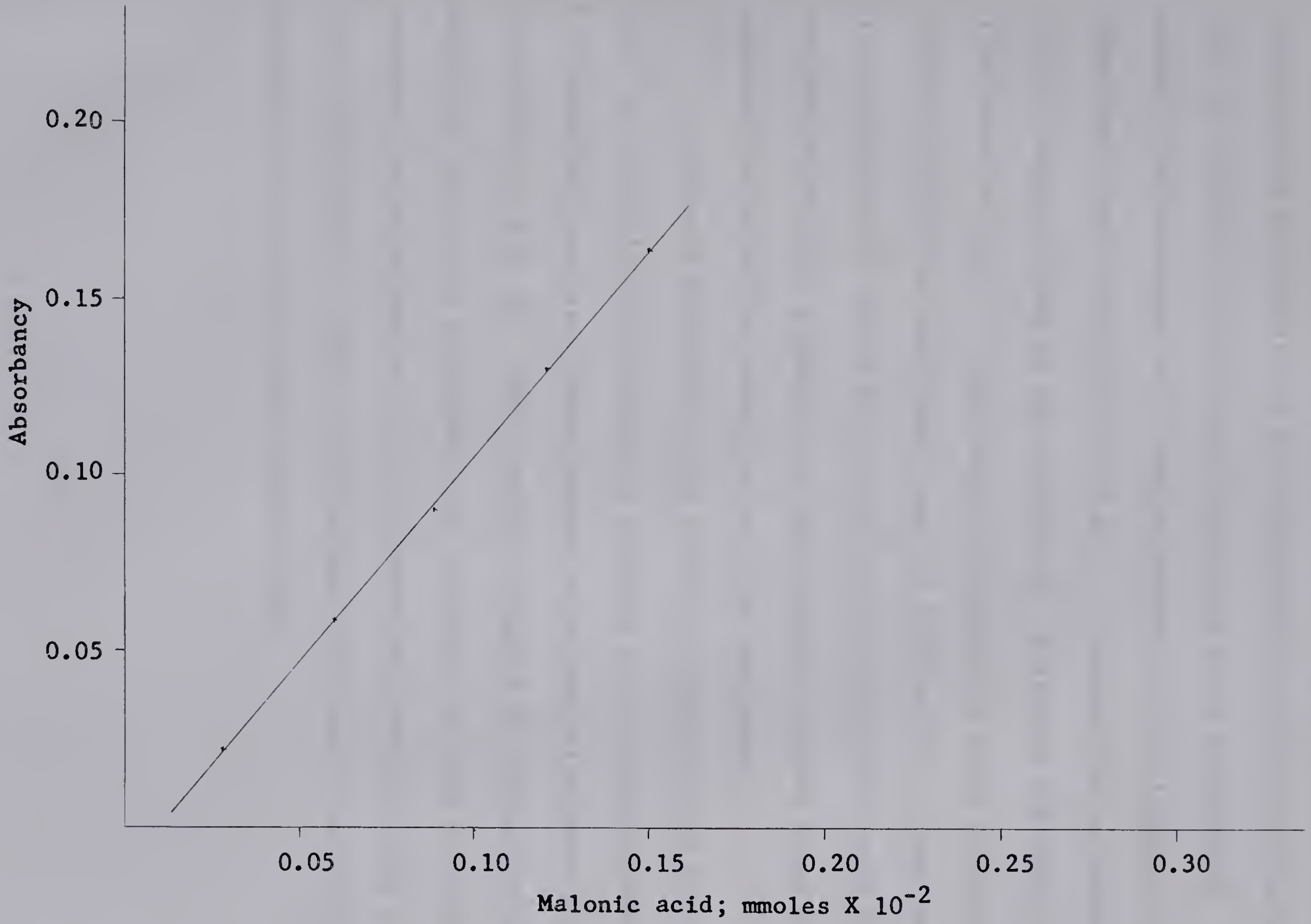


Fig. IX. Absorbancy at 242 mμ of Malonic Acid Complexed with Benzidine Hydrochloride and Cuprous Ion

The progress of the Snell reaction among β-alanine, pyridoxal hydrochloride and cupric ion was confirmed by quantitative determinations (102) of pyridoxal hydrochloride before and after autoclaving. For this purpose, the absorbancy of a complex between pyridoxal hydrochloride and ethanolamine was determined at 365 $m\mu$. A representative standard curve for the complex formed in the presence of β-alanine and cupric ion is illustrated in Fig. X. Experimental readings for which such curves were used were within the absorbancy range of 0.25 to 0.35. Experiments were also carried out in which one or two of these components were absent; standard curves were prepared for each separate system, since solution components affected the absorption at this wavelength.

A carbon-14 quenching curve (136) for radioactivity determinations by scintillation counting in a system of 2,5-diphenyloxazole and $2,2^1$-paraphenylene bis-5 phenyloxazole in toluene with Cab-o-sil is illustrated in Fig. XI. The curve was prepared by adding increasing amounts of acetone to fluor-Cab-o-sil mixtures, in each of which were suspended the scrapings of a spot of known radioactivity from a thin layer chromatogram. Hence the percentage efficiency of counting was determined in relation to the channel ratio.

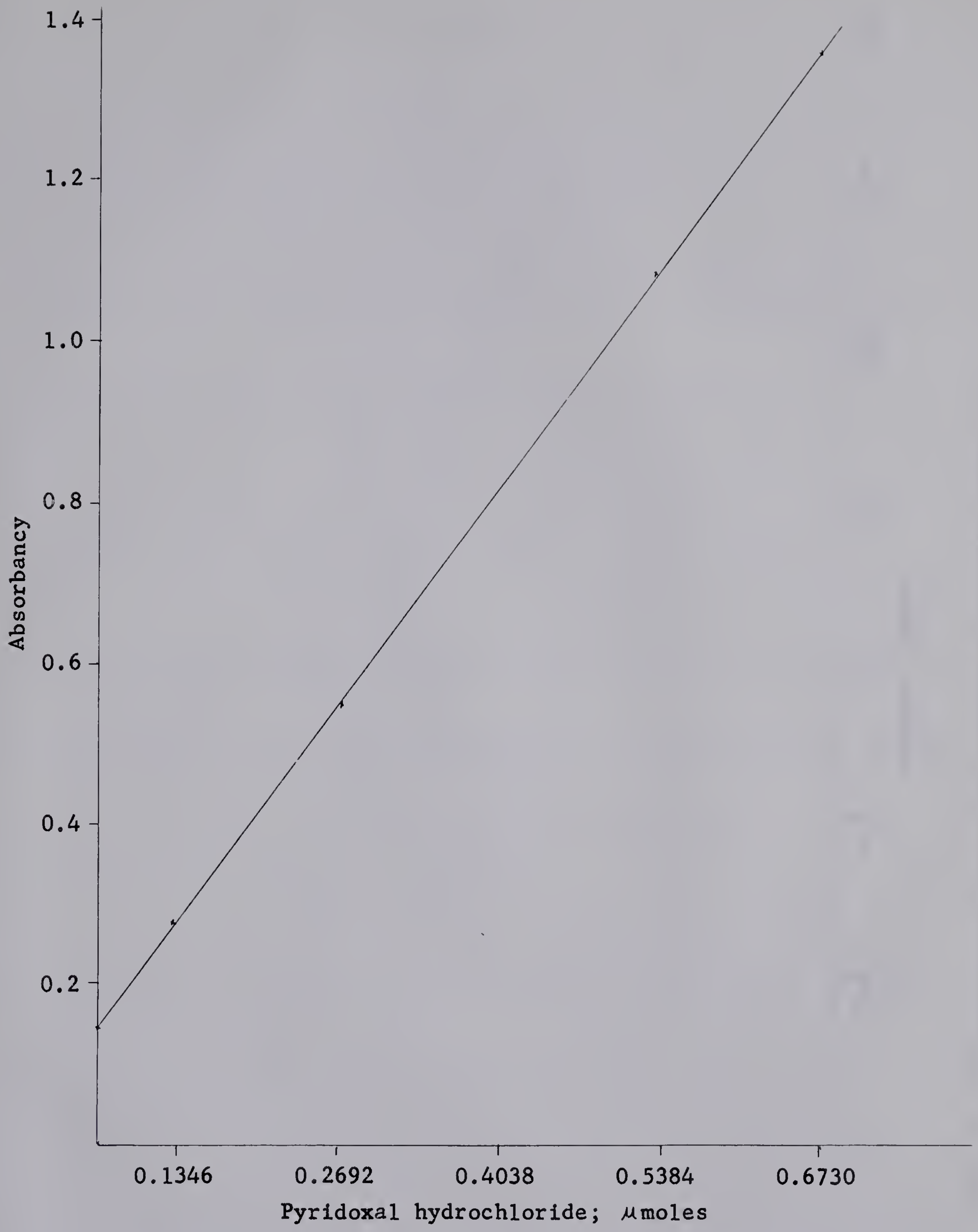


Fig. X. Absorbancy at 365 mμ of Pyridoxal Hydrochloride Complexed with Ethanolamine in the presence of β-alanine and Cupric Ion

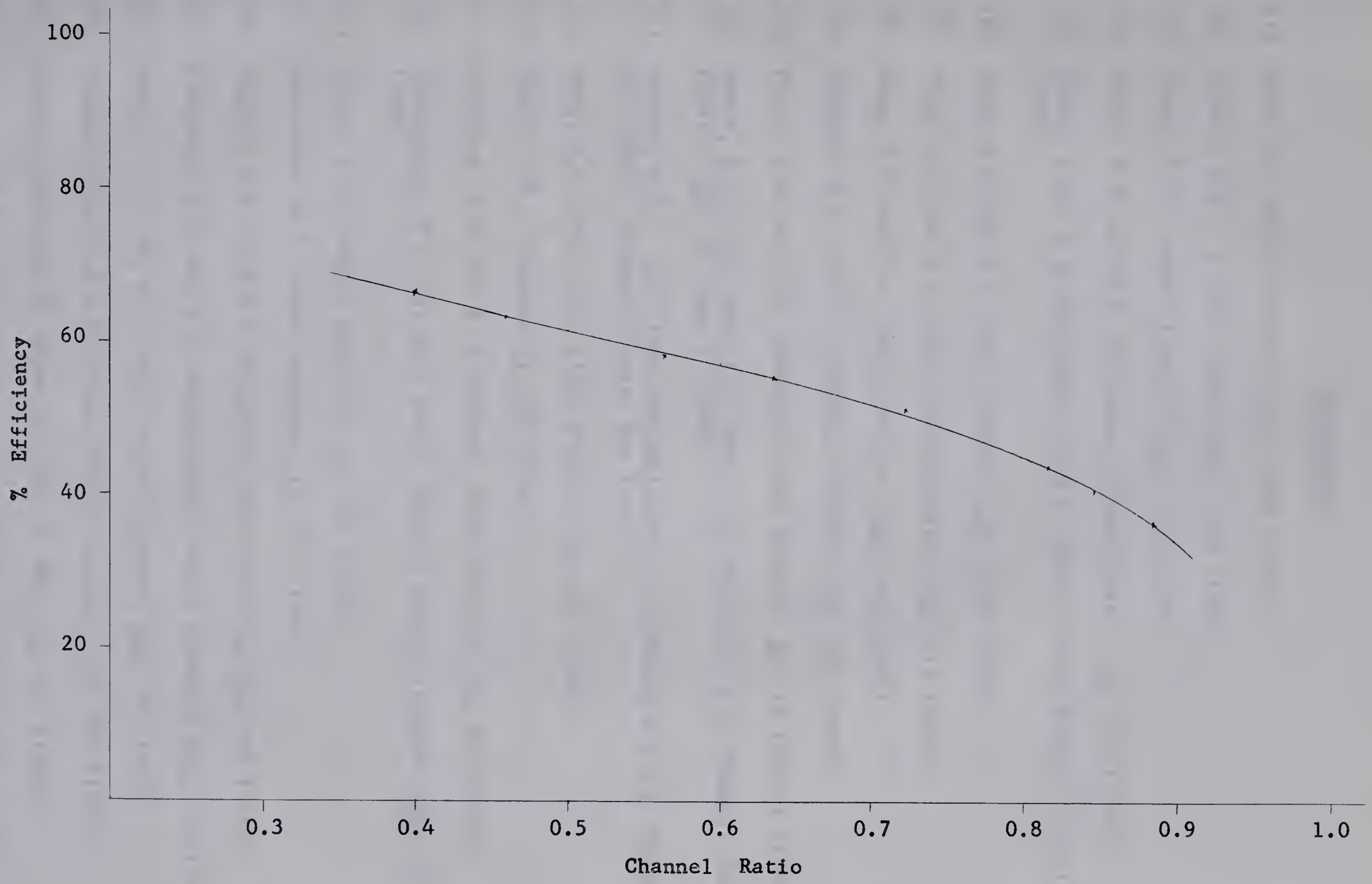


Fig. XI. ^{14}C-Quenching Curve for Toluene Fluor with Cab-o-sil

REFERENCES

(1) Gane, R., Nature (London) 134, 1008 (1934).

(2) Jansen, E.F., J. Biol. Chem. 238, 1552 (1963).

(3) Young, R.E., Nature (London) 205, 1113 (1965).

(4) Pratt, H.K. and W.B. McGlasson, Plant Physiol. 39, 120 (1964).

(5) Lyons, J.M., W.B. McGlasson and H.K. Pratt, Plant Physiol. 37, 31 (1962).

(6) Burg, S.P. and E.A. Burg, Science 152, 1269 (1966).

(7) Burg, S.P. and E.A. Burg, Plant Physiol. 37, 179 (1962).

(8) Burg, S.P. and E.A. Burg, Bot. Gaz. 126, 200 (1965).

(9) Spencer, M.S., Can. J. Biochem. Physiol. 36, 595 (1958).

(10) Pratt, H.K. and J.D. Goeschl, Plant Physiol. 39, IX (Supp.) (1964).

(11) Maxie, E.C., H.L. Rae, I.L. Eaks, S.E. Batal and N.F. Sommer, Plant Physiol. 39, IX (Supp.) (1964).

(12) Jansen, E.F., In "Plant Biochemistry", (J. Bonner and J.E. Varner), 643 (1965), Academic Press, New York.

(13) Burg, S.P., Ann. Rev. Plant Physiol. 13, 265 (1962).

(14) Biale, J.B., Science 91, 458 (1940).

(15) Spalding, D.H. and M. Lieberman, Plant Physiol. 40, 645 (1965).

(16) Sprayberry, B.A., W. Hall and C. Miller, Nature (London) 208, 1323 (1965).

(17) Biale, J.B., Advan. Food Res. 10, 293 (1960).

(18) Nickerson, W.J., Arch. Biochem. 17, 225 (1948).

(19) Dimond, A.E., and P.E. Waggoner, Phytopathology 43, 663 (1953).

(20) Freebairn H.T. and I.W. Buddenhagen, Nature (London) 202, 313 (1964).

(21) Morgan, P.W. and W.C. Hall, Nature (London) 201, 99 (1964).

(22) Spencer, Mary and A.O. Olson, Nature (London) 205, 699 (1965).

(23) Spencer, Mary and M. Meheriuk, Can. J. Bot. 42, 337 (1964).

(24) Abeles, F.B. and B. Rubinstein, Biochim. Biophys. Acta 93, 675 (1964).

(25) Pratt, H.K., Plant Physiol. 29, 16 (1954).

(26) Shah, J. and E.C. Maxie, Physiologia Plantarum 18, 1115 (1965).

(27) Chandra, G.R. and M.S. Spencer, Nature (London) 197, 366 (1963).

(28) Gibson, M.S., Biochim. Biophys. Acta 78, 528 (1963).

(29) Chandra, G.R. and M.S. Spencer, Biochim. Biophys. Acta 69, 423 (1963).

(30) Kokanov, M.T., Voprosy Med. Khim. 6, 158 (1960); Chem. Abst. 56, 1900 (1962).

(31) Spencer, Mary, Can. J. Biochem. Physiol. 34, 1261 (1956).

(32) Sacher, J.A., Nature (London) 195, 577 (1962).

(33) Baar, J.R. and M. Workman, Plant Physiol. 39, 540 (1964).

(34) Akamine, E.K., Science 140, 1217 (1963).

(35) Smith, W.H., D.F. Meigh and J.C. Parker, Nature (London) 204, 92 (1964).

(36) Pratt, H.K. and W.B. McGlasson, Plant Physiol. 39, 128 (1964).

(37) Rubinstein, B. and F.B. Abeles, Bot. Gaz. 126, 255 (1965).

(38) Hall, W.C. and P.W. Morgan, Editions du Centre National de la Recherche Scientifique 15, 727 (1964).

(39) Chandra, G. Ram and Mary Spencer, Nature (London) 194, 361 (1962).

(40) Biale, J.B., R.E. Young and A.J. Olmstead, Plant Physiol. 29, 168 (1954).

(41) Spencer, Mary, In "Plant Biochemistry" (J. Bonner and J.E. Varner), 817 (1965), Academic Press, New York.

(42) Wooltorton, L.S.C., J.D. Jones and A.C. Hulme, Nature (London) 207, 999 (1965).

(43) Kidd, F. and C. West, Plant Physiol. 20, 467 (1945).

(44) Finch, A.H., Proc. Amer. Soc. Hort. Sci. 34, 74 (1936).

(45) Sorber, D.G. and M.H. Kimball, U.S.D.A. Technic. Bull. 996, 1 (1950).

(46) Pratt, H.K. and M. Workman, Proc. Amer. Soc. Hort. Sci. 81, 467 (1962).

(47) Lyons, J.M. and H.K. Pratt, Proc. Amer. Soc. Hort. Sci. 84, 491 (1964).

(48) Lyons, J.M. and H.K. Pratt, Arch. Biochem. Biophys. 104, 318 (1964).

(49) Olson, A.O. and Mary Spencer, Proc. Can. Soc. Plant Physiol. 7, 19 (1966).

(50) Hale, W.S., S. Schwimmer and E.G. Bayfield, Cereal Chem. 20, 224 (1943).

(51) Vacha, G.A. and R.B. Harvey, Plant Physiol. 2, 187 (1927).

(52) Marinos, N.G., J. Expt. Bot. 11, 227 (1960).

(53) Abeles, F.B. and B. Rubinstein, Plant Physiol. 39, 963 (1964).

(54) Abeles, F.B., Plant Physiol. 41, 585 (1966).

(55) Chipman, C.N., Curr. Res. Anesth. Analg. 10, 206 (1931).

(56) Craft, C.C., Plant Physiol. 35, VII (Supp.) (1960).

(57) Spencer, Mary and M. Meheriuk, Nature (London) 199, 1077 (1963).

(58) Burg, S.P. and K.V. Thimann, Plant Physiol. 35, 24 (1960).

(59) Spencer, M.S., Can. J. Biochem. Physiol. 37, 53 (1959).

(60) Burg, S.P., Arch. Biochem. Biophys., 84, 543 (1959).

(61) Burg, S.P. and K.V. Thimann, Arch. Biochem. Biophys. 95, 450 (1961).

(62) Wang, C.H., A. Persyn and J. Krackov, Nature (London) 195, 1306 (1962).

(63) Burg, S.P. and E.A. Burg, Nature (London) 203, 869 (1964).

(64) Burg, S.P. and E.A. Burg, Science 148, 1190 (1965).

(65) Gibson, M.S. and R.E. Young, Nature (London) 210, 529 (1966).

(66) Phan Chan Ton, "Contribution a l'étude de la production d'éthylene par le Penicillium digitatum Sacc.," Doctoral Dissertation, l'Univ. de Paris, (1961).

(67) Gibson, M.S., Nature (London) 202, 902 (1964).

(68) Wang, C.H., D.W. Jacobsen and F.S. Tanaka, Fed. Proc. 23, 224 (1964).

(69) Spalding, D.H. and M. Lieberman, Plant Physiol. 38, lviii (Supp.) (1963).

(70) Jacobsen, D.W., and C.H. Wang, Plant Physiol. 40, XIX (Supp.) (1965).

(71) Jansen, E.F., J. Biol. Chem. 239, 1664 (1964).

(72) Lieberman, M. and L.W. Mapson, Plant Physiol. 39, IX (Supp.) (1964).

(73) Rich, S. and J.G. Horsfall, In "Metabolic Inhibitors" (R.M. Hochster and J.H. Quastel) 271, vol II (1963), Academic Press, New York.

(74) Abeles, F.B., Nature (London) 210, 23 (1966).

(75) Lieberman, M., A.T. Kunishi, L.W. Mapson, and D.A. Wardale, Biochem. J. 97, 449 (1965).

(76) Lieberman, M., A. Kunishi, L.W. Mapson and D.A. Wardale, Plant Physiol. 41, 376 (1966).

(77) Spencer, M.S., Nature (London) 184, 1231 (1959).

(78) Meheriuk, M. and Mary Spencer, Nature (London) 204, 43 (1964).

(79) Meheriuk, Michael, "Some Biochemical Studies on the Production of Ethylene by a Particulate Fraction from Ripening Tomatoes", Doctoral Dissertation, University of Alberta (1965).

(80) Lieberman, M. and C.C. Craft, Nature (London) 189, 243 (1961).

(81) Burg, S.P. and E.A. Burg, Nature (London) 191, 967 (1961).

(82) Lieberman, M. and P. Hochstein, Science 152, 213 (1966).

(83) Hayaishi, O., Y. Nishvzaka, M. Tatibana, M. Takeshita and S. Kuno, J. Biol. Chem. 236, 781 (1961).

(84) Goldfini, H. and E.R. Stadtman, J. Biol. Chem. 235, 2238 (1960).

(85) Rendina, G., and M.J. Coon, J. Biol. Chem. 225, 523 (1957).

(86) Hatch, M.D. and P.K. Stumph, Arch. Biochem. Biophys. 96, 193 (1962).

(87) Fritzson, Per and K.F. Nakken, Acta Chem. Scand. 10, 161 (1956).

(88) Campbell, L.L. Jr., J. Bacteriol. 73, 225 (1957).

(89) Campbell, J.W., Biochem. J. 77, 105 (1960).

(90) Burnes, R.L. and A.W. Naylor, Plant Physiol. 37, 171 (1962).

(91) Tsai, C.S. and B. Axelrod, Plant Physiol. 40, 39 (1965).

(92) Fritzson, Per, Acta Chem. Scand. 13, 2124 (1959).

(93) Chandra, G. Ram, "Studies on Ethylene Production by Sub-Cellular Fractions", Doctoral Dissertation, Univ. of Alberta, (1963).

(94) Young, R.E., H.K. Pratt and J.B. Biale, Anal. Chem. 24, 551 (1952).

(95) Penefsky, H.S., M.E. Pullman, A. Datta and E. Racker, J. Biol. Chem. 235, 3330 (1960).

(96) Roberts, H.R. and W. Bucek, Anal. Chem. 29, 1447 (1957).

(97) Snyder, F. and N. Stephens, Anal. Biochem. 4, 128 (1962).

(98) Draganic, Z.D., Anal. Chim. Acta 28, 394 (1963).

(99) Burchfield, H.P. and E.E. Storrs, "Biochemical Applications of Gas Chromatography" 574 (1962), Academic Press, New York.

(100) Stark, J.B., A.E. Goodman and H.S. Owens, Anal. Chem. 23, 43 (1951).

(101) Snell, E.E., J. Amer. Chem. Soc. 67, 194 (1945).

(102) Metzler, D.E. and E.E. Snell, J. Amer. Chem. Soc. 74, 979 (1952).

(103) Akazawa, T. and H. Beevers, Biochem. J. 67, 115 (1957).

(104) Opik, H., J. Expt. Bot. 16, 667 (1965).

(105) Lusena, C.V. and C.M.S. Dass, Can. J. Biochem. 44, 775 (1966).

(106) Breedis, C., J. Exptl. Med. 76, 221 (1942).

(107) Morton, R.K., In "Methods in Enzymology" (S.P. Colowick and N.O. Kaplan) 1, 46 (1955) Academic Press, New York.

(108) Braunstein, A.E., In "The Enzymes" (P.D. Boyer, H. Lardy, and K. Myrback) 2, 126 (1960), Academic Press, New York.

(109) Kupiecki, F.P. and M.J. Coon, J. Biol. Chem. 229, 743 (1957).

(110) Roberts, E., P. Ayengar and I. Posner, J. Biol. Chem. 203, 195 (1953).

(111) Roberts, E. and H. M. Bregoff, J. Biol. Chem. 201, 393 (1953).

(112) Pattee, H.E. and L.M. Shannon, Bot. Gaz. 126, 179 (1965).

(113) Vagelos, P.R. and J.M. Earl, J. Biol. Chem. 234, 2272 (1959).

(114) Strausz, O.P. and H.E. Gunning, J. Amer. Chem. Soc., 84, 4080 (1962).

(115) Stinson, R.A. and M.S. Spencer, Unpublished data.

(116) Goldman, P. and P.R. Vagelos, In "Comprehensive Biochemistry" (M. Florkin and E.H. Stotz) 15, 71 (1964), Elsevier Publishing Company.

(117) Jaenicke, L. and F. Lynen, In "The Enzymes", (P.D. Boyer, H. Lardy and K. Myrback) 3, 3 (1960), Academic Press, New York.

(118) Bender, M.L. and R. Breslow, In "Comprehensive Biochemistry" (M. Florkin and E.H. Stotz) 2, 90 (1962), Elsevier Publishing Company.

(119) Mizusaki, S., M. Noguchi and E. Tamaki, Arch. Biochem. Biophys. 105, 599 (1964).

(120) Cossins, E.A. and H. Beevers, Plant Physiol. 38, 375 (1963).

(121) Lieberman, M. A.T. Kunishi, L.W. Mapson and D.A. Wardale, Plant Physiol. 40, XIX (Supp.) (1965).

(122) Vagelos, P.R., J.M. Earl and E.R. Stadtman, J. Biol. Chem. 234, 490 (1959).

(123) Giovanelli, J. and P.K. Stumpf, J. Amer. Chem. Soc. 79, 2652 (1957).

(124) Giovanelli, J. and P.K. Stumpf, J. Biol. Chem. 231, 411 (1958).

(125) Den, H., W.G. Robinson and M.J. Coon, J. Biol. Chem. 234, 1666 (1959).

(126) Vagelos, P.R., J. Biol. Chem. 235, 346 (1960).

(127) Den, H. and H.P. Klein, Biochim. Biophys. Acta 49, 429 (1961).

(128) Formica, J.V. and R.O. Brady, J. Amer. Chem. Soc. 81, 752 (1959).

(129) Young, R.H. and L.M. Shannon, Plant Physiol. 34, 149 (1959).

(130) Giovanelli, J. and P.K. Stumpf, Plant Physiol. 32, 498 (1957).

(131) Duperon, R., Compt. Rend. 251, 260 (1960).

(132) Duperon, R., Compt. Rend. 252, 1195 (1961).

(133) Duperon, R., Compt. Rend. 256, 2906 (1963).

(134) International Critical Tables, Vol. VII, 245 (1930).

(135) Ogg, C.L., J. Assoc. Offic. Agric. Chem. 43, 689 (1960).

(136) Nuclear Chicago Liquid Scintillation Manual, page 83, (1965).

(137) Meigh, D.F., Nature. (London) 196, 345 (1962).

B29861